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Doing something for the centre

THERE is something distinctly unnerving about writing about the British General Election before it happens yet being published only after it is all over. The one thing that the last two elections have clearly indicated is that it would be the height of folly to assume even two days before the event that any particular party had won. The most recent opinion polls unanimously point to a Labour victory with 42% of the vote compared with the Conservatives' 35% and the Liberals' 19% and if this pattern persisted until polling day a Labour government would be returned with a majority of at least fifty over all other parties. Certainly the pattern has shown a stability over the past few weeks which reflects the inability of any political party to make a major impact on the uncommitted, but there is a feeling that there is still all to fight for.

The issues before the electorate are more serious than they have been at any time in the recent past, and the party positions are probably as clear-cut and distinguishable as they have ever been. And yet it is this very clearness of division which has made the choice for anyone who gives some thought to his decision an extremely difficult one to make this time. For separation on the issues, particularly on how to handle inflation and on how to fortify British industry, inevitably means bringing out that perennial skeleton in the cupboard — class warfare. Both Labour and Conservatives have, of course, their mechanisms for moderation; the Labour party's social contract with the unions looking at present deservedly more like an election-winner than the Conservative pledge to create a government of national unity. And yet the social contract, a sort of Swedish arrangement with the unions to work for more equality in exchange for moderation in wage claims is not universally enthused over by the unions, particularly those which represent the more-than-equal at present. And if anything there is more socially divisive electioneering from Labour than there was in February, and there have even been threats of industrial chaos this winter if Labour does not win.

Not that the Conservative party has actually been proposing policies which seem to be aimed at picking up the floating voter who might be disturbed by the threatening noises from the left, but fears that old-style Conservatives would get the country back into confrontation politics between government and unions. There is as yet no sign that old-style Toryism is being replaced by something more of the centre.

And yet it is pursuit of the centre which both of the present major political parties ought to be engaged in at

a time of deep crisis if they seek electoral success; surely the McGovern experience ought to have taught moderates the world over that lesson. In any country with a less perverse electoral system it would be ridiculous that the support of 42% of the populace should give an unfettered mandate to a party, and it is high time that if the two major parties do not move to the middle ground, life should be made easier for the Liberals—who have been occupying it without success for so long.

The dilemma for the voter contemplating the Liberals has always been the taunt that a vote for them was a vote wasted, and yet even against this they have made steady progress until this election. It is unlikely that they will make much more headway now for rather obvious reasons. Their policies are widely regarded as attractive, sensible and likely to unite the country but their abilities are bound to be suspect whilst they have a mere handful in parliament but an army of untried hopefuls outside. There are many able politicians of the centre at present in the two main parties who, one suspects, would happily defect to the Liberals given some job prospects. The party finds itself in the bind of having amassed the support of a fifth of the electorate, the potential for more and yet, without being given some sort of break, it probably will be incapable of going above the critical size (at about 28% support the party would probably have a hundred or so members in parliament).

The case for letting some fresh air in to the British parliament is strong and it is only just that a party of the centre with a broad support should provide much of this fresh air and help towards realigning British party politics in a more healthy way. But this is unlikely to occur in the near future unless electoral reform is taken seriously by one of the two main parties. The question of which is the best of many systems of representatives tried elsewhere should not be allowed to bog down the central issue of the need for reform. And if in the next few years neither party comes to terms with electoral reform, it will be incumbent on the more thoughtful and moderate to lobby intelligently for social justice at the very place which claims to dispense social justice.

A hundred years ago



An Anagram

THE practice of enclosing discoveries in sealed packets and sending them to Academies, seems so inferior to the old one of Huyghens, that the following is sent you for publication in the old conserved form :—

A⁸C³DE¹²F⁴GH¹⁶I⁶L³M³N⁶O⁶P
R⁴S³T⁴U⁶V²WXY²

WEST

From *Nature*, 10, 480, October 15, 1874.



All this and heavens too



On October 16 the Anglo-Australian 150-inch telescope is being inaugurated at Siding Spring in New South Wales. Together with the recent announcement of plans for an infrared flux collector in Hawaii, the imminent retirement of the Director of the Royal Greenwich Observatory and of the Astronomer Royal for Scotland, and with the concept of a Northern Hemisphere Observatory very much alive we are clearly at the beginning of a new era in British astronomy. John Gribbin has been talking to Dr Alan Hunter and Professor F. G. Smith, of the RGO, and Dr V. C. Reddish, of the Royal Observatory Edinburgh, about future prospects in the light of these developments. Picture: dome of UK 48-inch Schmidt telescope at Siding Spring.

IN terms of optical astronomy, Britain has been behind the United States since the beginning of this century. The 60-inch telescope completed some 70 years ago and other telescopes on good sites within the home territory of the United States made this inevitable; and modern astrophysics needs big telescopes. Only 130 years ago, of course, the biggest telescope in the world was in Ireland. But that was no consolation to the bright youngsters in Britain who were interested in astronomy in the 1920s and later. The natural reaction of such people was to move into theoretical work, where thanks to Eddington and others Britain became as pre-eminent as the United States was in observational work.

And that, of course, led to a still more lopsided growth of the subject in Britain. The next generation followed where these pioneers had led and achieved fame, and what could have been a very unhealthy situation was saved only by the advent of radio-astronomy.

In recent years there have sometimes been bitter attacks on the administrators of British science for failing to prevent Britain slipping behind in terms of optical telescopes. But much of this criticism, made only now with the benefit of hindsight, seems a little

harsh when we recall the conditions which prevailed, say, 30 years ago. Dr Alan Hunter, who retires as Director of the Royal Greenwich Observatory (RGO) next year recalls that in 1947 it was still something of an adventure to travel to South America to view a solar eclipse. In those days, the aircraft still flew south on the first leg of the journey, in order to cross the Atlantic at its narrowest point. Yet the decision to build the Isaac Newton Telescope (INT) in Sussex was taken in 1946; could the planners really have been expected then to appreciate how jet travel would revolutionise astronomy, opening up the prospect of British telescopes being run effectively even when built in Australia or Hawaii?

The experience now being gained with the Anglo-Australian Telescope (AAT) and the UK Schmidt camera on the same site illustrates how rapidly things have changed over the past decade. Dr V. C. Reddish, of the Royal Observatory Edinburgh (ROE) has been closely associated with both projects, and is at present on the management committee of the AAT. The Schmidt camera has now been operating for a year, and he says that the first papers reporting observations made with the instrument will be published soon. The importance of this

camera, and the reason for siting it with the AAT, is that it produces a wide angle view of the sky, covering twelve times the Moon's diameter on one plate. Essentially, such a camera is used for survey work while the more powerful but narrower angle optics of the 150-inch will be used to investigate interesting sources revealed by the survey. The Schmidt plates are also of great interest to radioastronomers who wish to identify the sources they have found.

In the northern hemisphere, the Palomar Sky Survey (obtained with a 48-inch Schmidt camera) has provided the basic sky atlas for astronomers over twenty years or so. But there has never been a comparable survey of the southern sky.

This is being rectified by the UK Schmidt and by a similar instrument at the European Southern Observatory in Chile (ESO). Each camera is taking a set of plates covering the whole southern sky, but at two different wavelengths. Together, these plates make up an atlas which enables astronomers to pick out many interesting objects—such as possible QSOs—from their relative brightness at two frequencies. Reddish says that the operation of the joint survey with ESO has been “co-operative, pleasant and efficient”. ESO plate copying facilities in Geneva are being used for both ESO and UK Schmidt plates, and this specific area of collaboration typifies the way astronomy has developed as an international science.

The UK Schmidt has given British astronomers experience of both international cooperation and of the building and running of a really big telescope. Of 800 plates produced in the first year of operation, more than 100 have been accepted for the southern sky survey. This reflects a wish to maintain high standards, since plates once accepted for the survey will be used by many people for many years, together with a reluctance to seek perfection, which would make the task impossibly long.

Even so, thanks to the advances made since the Palomar Sky Survey was carried out in the north, the new Schmidt is producing plates which are a distinct improvement in quality over any previous survey. Thanks partly to the use of a new photographic emulsion (known as IIIaJ) and a new technique for treating plates before exposure by soaking them in oxygen-free environment (*Nature*, **249**, 22: 1974), the plates record all celestial objects down to magnitude 23 with an exposure of 40 minutes. This means that twice as many radio sources can be identified as on comparable Palomar Sky Survey plates, and that radioastronomers can even tell

what type of galaxy their sources are associated with at magnitudes which would not even be recorded on Palomar plates.

There is no direct association between the building of the Schmidt and of the AAT, but inevitably some expertise must rub off from one committee to another, since some people, like Reddish, have been involved in both projects. Reddish points out that although the AAT will be the third biggest telescope in the world it has "superb" optics and that, together with the improvement of instrumentation over the past 30 years, means that it could well be even more powerful in practice than the 200-inch, just as the 48-inch UK Schmidt is more powerful than its older counterpart at Palomar. Already there is great demand for time on the AAT, and even some very good projects will inevitably fail to receive an allocation.

The attitude of the Science Research Council (SRC) certainly seems more realistic than they have sometimes been given credit for. Accepting that a capital investment of £1 million must be used efficiently, they are prepared to fly people out to Australia to work with the new telescope two or three times a year. And there may well be more large capital investment in the offing, again at sites far from Britain.

Already the decision has been made to go ahead with construction of a 3.8-m infrared flux collector in Hawaii (*Nature*, **250**, 617; 1974), and a decision will be taken in the new year on whether to build a UK Northern Hemisphere Observatory (NHO). This idea has been in the wind for several years, but should now receive the go ahead. France, Germany and Italy are all planning their own large telescopes, and although this activity might seem to run counter to the spirit of international cooperation in astronomy that is not really the case.

According to Dr Reddish, it is not efficient to share construction of telescopes and observatories too widely—the technology of interfaces are difficult enough to cope with without the worry of international management interfaces. But it does make sense to share sites, services and overheads. Through that kind of sharing joint use of the instruments will inevitably follow.

All that, together with the infrared telescope decision, must make Hawaii one of the favourite contenders for the site of the NHO. That site is really a little too high, since it becomes difficult to breathe on top of the Hawaiian mountains. But otherwise it seems ideal, with a US observatory already there and obvious possibilities of sharing the running expenses. With such an observatory—in Hawaii or elsewhere—Britain could well, in fact, lead the world in observational astronomy for

the rest of this century.

That would certainly be a remarkable development. But it reflects the growing awareness that astrophysics is rapidly becoming the big science of the present day. Both Reddish and Professor F. G. Smith, who has just left the University of Manchester to go to the RGO as Deputy Director, and eventually to succeed Dr Hunter as Director, commented on the high standard of people entering astronomy today. There is no evidence of a drift away from the sciences here; in fact, the very best students now are of a higher calibre than ever before.

This is understandable. It is now clear that the next great advances to be made in physical science are likely to centre on problems of high energy and the study of gravitation. And these problems cannot be tackled in the laboratory. Nuclear astrophysics, cosmic ray studies, and information about energetic sources obtained at all parts of the spectrum are the 'laboratory' sciences of the immediate future. It is no wonder, then, that the first class physical scientists today are attracted primarily to astronomy.

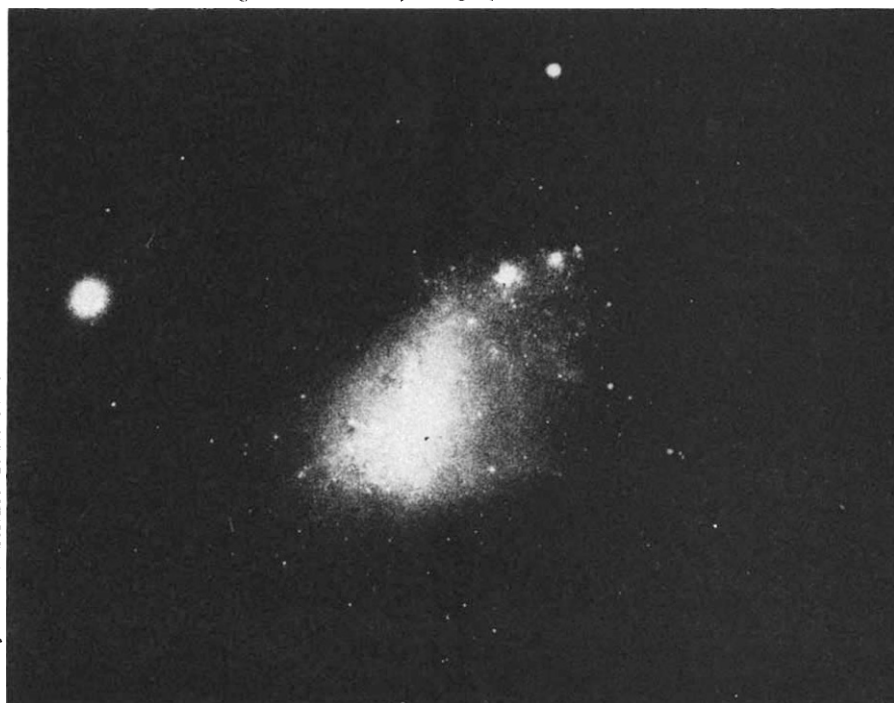
Indeed, Smith's move from Manchester to the RGO is one symptom of how astronomy's face is changing. Smith emphasises that he is an observer, not a theorist, but that there are no barriers between different astronomical observers any more. With modern instrumentation, whatever kind of 'electromagnetic radiation bucket' is used to collect signals, the job of the observer is really one of analysing electric impulses. So it is misleading to talk about 'radio', 'optical' or 'infrared' astro-

nomers; as Smith says, "we are all just 'astronomers' now".

Even so, there must be a good reason for Smith to move to the RGO at the present time, and it seems that the lure of the NHO has played a large part in this. The RGO has the job of creating and operating the NHO (assuming it receives the go ahead), and could well become the most important astronomical centre not just in Europe but in the world—indeed, the way science is progressing at present, one might well envisage the RGO becoming the key scientific centre in the world. It is small wonder that Smith should be attracted by the challenging job of acting as Director of the RGO for the exciting years ahead.

Both the RGO and the ROE have university links, the ROE a long established relationship with the University of Edinburgh and the RGO a more recently established but important link with the University of Sussex. The RGO also provides summer schools in astronomy for university students from all over Britain, and these are so popular that only one applicant in five can be accommodated. In Edinburgh, when a job on the UK Schmidt was advertised recently there were ten applicants, nine of whom had first class degrees and were short listed. This is unprecedented in Reddish's experience; astrophysics is now getting the pick of the research students in the same way that nuclear physics did 50 years ago. Looking at the progress made in nuclear physics since the 1920s, that simple statistic, as much as anything else, points to an exciting time for British astronomy in the next 25 years.

The Lesser Magellanic Cloud: photographed through the UK Schmidt telescope.



Courtesy of the Science Research Council.

international news

Nature published two replies (June 14, July 5) to the letter from Dr Gerschenfeld and his colleagues (March 29) dealing with the dramatic conditions of the Chilean academic community. These replies support the military junta, minimise the abrogation of civil liberties and democratic government in Chile, and suggest that Dr Gerschenfeld and his colleagues were not properly informed. Coming from supposedly well-informed Chileans, they leave the impression that the situation there has indeed been over-dramatised. In fact both replies are studded with misleading and tendentious statements we feel obliged to correct. Most of us worked and lived in Chile under the government of Dr Salvador Allende and during the military coup of September 11. We feel qualified to write about conditions in the Chilean academic community at least as much as Admiral Swett, who had no relation with academic life before his appointment as rector of the Catholic University, or Professor Eyzaguirre who has lived for the past 17 years in the United States.

Admiral Swett admits in his letter that he was nominated rector by the military junta, which is extralegal. To minimise this abuse of power he adds that he was confirmed by the head of the Catholic Church. However, the Catholic bishops of Chile in a collective declaration of April 1974 entitled "The reconciliation in Chile" have publicly stated their concern for "... arbitrary arrests or imprisonment by ideological motives ..." and declared that Chileans are "living in a climate of insecurity and fear". We believe that the head of the church had no alternative to accepting the nomination of Admiral Swett. Professor Eyzaguirre repeated this and other remarks, but in a very different style. He spent half of his letter discrediting the Allende government and justifying the military coup, expounding the official statements of the military junta. This is a general polemic, and irrelevant to the specific charges presented in the Dr Gerschenfeld's letter and under International News in *Nature*, May 3. Few factual statements were challenged in the two "replies from Chile". We wish to comment on those few challenges since they definitely distort the reality.

"Students at some universities had to reregister. Students who did not qualify as such in the traditional sense (...) were dismissed. This weeded

out the purely political 'students,' ...". In fact, all students at five out of six universities had to reregister. 20,000 students were dismissed. Since more than a thousand lacked a few credits or a final examination to obtain their degrees and had entered the university two to four years before Allende was elected president, it is difficult to see them as purely political "students".

"The Faculty went through the same screening procedure. Those who

In reply to recent letters in *Nature* from supporters of the Chilean junta, twenty-six Chilean scientists have signed this joint statement on the condition of academics in the country. Their names have been supplied to the Editor.

did not qualify for lack of degrees or for being exclusively political activists were dismissed," wrote Professor Eyzaguirre. More than 60% of the Faculty of the Technical University, almost the whole Faculty of the schools of Anthropology, Psychology, Sociology and Economics at the University of Chile, Concepcion and others, and most of the members of the prestigious Department of Public Health of the School of Medicine at the University of Chile would then be exclusively political activists. Admiral Swett said "They were dismissed because they simply abandoned their jobs. ...". Many of us were indeed dismissed for abandonment of our jobs but it should be known that we were absent because of imprisonment.

"Very few fell either immediately after or during the coup because, allegedly, they were caught either shooting at soldiers, carrying arms or actively participating in the organisation of revolutionary activities." We and almost every colleague know that professors Mario Ramirez, Jorge Pena and Enrique Paris neither shot at soldiers nor carried arms; we must infer that they were executed because of "organisation of revolutionary activities"; a very broad charge since the government of Allende was categorised as revolutionary.

"There are charges against some of these people and if they return they will face legal prosecution. But prose-

cution is one thing and persecution another." It is general knowledge that legal procedures have been suspended in this instance. Dr Edgardo Enriquez, minister of education and former rector of the University of Concepcion; Mr Enrique Kirberg, rector of the Technical University; Mr Galo Gomez, president of CONICYT; Mr Raul Iriarte, secretary of CONICYT, and many other less known members of the academic community have been imprisoned from September 1973 with no formal charges and no trial date.

"Some academicians have had trouble with the police because of denunciation by neighbours or colleagues. These people have been investigated ...". Investigation consisted in arrest and interrogation usually with physical mistreatment. Many of us experienced this and it was clear to us that this was purely political persecution.

"The appointments ... (of retired senior armed forces officers as rectors) ... would not be too far from practices that are not unknown in the United States." The appropriate parallel to the Chilean situation would be the appointment of military presidents in every American university, by the Pentagon.

"Chaotic finances which prevented normal university activities and the payment of adequate personnel salaries" (during Allende's government). There were indeed some financial problems for adequate scientific research but during this time the universities received their largest share in the history of the national budget. This policy should be compared with that of the junta which ordered every university to finance itself with no funds from the government by January 1976 while the share of the armed forces has been increased from under 10 to 40% of the national budget.

With regard to Chilean scientists remaining in Chile, we agree that they are not fascist (nothing close to that is said in Dr Gerschenfeld's letter) but people sincerely devoted in pursuing their research under very difficult circumstances. We believe, however, that the letters of Admiral Swett and Professor Eyzaguirre do not help them and are in fact unfair since these colleagues are not in a position to disagree. Chilean scientists remaining in Chile as well as those who will return are better helped by encouraging the return of at least academic freedom.

● THE COURT of inquiry into the explosion which killed 28 people and caused £70m damage at the Nypro chemical works in Flixborough, Lincolnshire, has been told that a temporary by-pass system in the cyclohexane unit was not tested at an appropriate pressure. According to counsel for the company (owned jointly by Dutch State Mines and the National Coal Board) it is now clear that the by-pass system was installed without an adequate design study, and that if such a study had been carried out, a different system would have been used. Most of the technical evidence has yet to be presented, and a recess is expected to allow for a consensus of expert specialist opinion. Reaching the consensus, however, will be difficult because of the quantity and complexity of the technical information likely to be available.

But there is no doubt that the findings of the inquiry will have profound implications for the chemical industry in Britain and elsewhere because of the fundamental safety issues which have been raised. The complexity of chemical plants and the huge reserves of stored energy they contain seem to have completely outrun the rather Victorian arrangements of the Factories Inspectorate—the 1974 Health and Safety at Work Act notwithstanding. The safety officer at the Flixborough site told the court that nothing on the scale of the explosion had ever been conceived or considered and that the rule book governing the safety of chemical plants needed rewriting in the light of events there.

The Health and Safety Commission now being set up is bound to look carefully at the results of the findings of the Flixborough Inquiry. As an independent commission it will bring together the existing government inspectorates covering nuclear installations, explosives, alkali works, mines, quarries and factories. The commission was proposed before the events at Flixborough but it comes at a time when a lead to industry is desperately needed. Already some large petrochemical companies have approached the Atomic Energy Authority Safety Commission for consultation on safety problems and there is a growing feeling, not least amongst the companies themselves, that the chemical industry should be governed by regulations at least as stringent as those of the Nuclear Installations Inspectorate.

● IN SPITE of recent inflammatory news reports, vaccination is still the best means of ensuring overall community protection from infectious diseases. This was the outcome of a report on

vaccination published by the Office of Health Economics (OHE) last week.

While diseases such as polio, diphtheria, tetanus and measles still present significant health hazards, OHE maintains that in some areas of the UK, only two-thirds or less of all children are adequately immunised. Thus to maintain public trust in vaccines available from the National Health Service, withdrawal of controversial vaccines such as whooping cough (pertussis) may become necessary in the near



Round Britain

future. It has been claimed that pertussis vaccine has caused brain damage in a small number of young infants, although there are no published figures to substantiate such an accusation. The OHE, however, recognises that an element of risk is associated with vaccination, but takes the view that provided the benefits far outweigh the risk then it is acceptable.

The study suggests that only a very small proportion (probably a few hundred) of the 50,000 to 60,000 severely mentally handicapped children in Britain owe their condition to immunisation by pertussis vaccine. But this small proportion has become of increased relative significance because of the fall in risks from other factors, such as infectious diseases. In an ideal national health care service, argues OHE, special provisions for the compensation of those damaged by vaccines should not have to be made because services for all the handicapped should be adequate. However, state compensation for vaccine-damaged people and their families may well draw attention to the poor provisions for the majority of brain-damaged people.

Improved living conditions, environmental health measures, new medicines and vaccination programmes, says the report, have reduced health risks in Britain from all known infectious diseases. In fact over the past 30 years, the number of child deaths from infectious diseases has dropped from about 10,000 to virtually none. Thus withdrawal of vaccination programmes which have outlived their usefulness should be considered.

Worldwide progress in smallpox immunisation has been so effective that in this country the risks associated with 'protection' outweigh the dangers of smallpox infection. The withdrawal of

routine smallpox immunisation in 1971 by the NHS is supported by the OHE, although here again there has been much criticism of this action mainly because of the occasional reappearance of the disease.

All the same there appears to be strong evidence to indicate that there is a positive benefit to be derived from all the vaccines currently in use by the NHS, the only possible exceptions being BCG and pertussis vaccines.

The attention which pertussis vaccine has received was criticised by OHE as being over dramatised, particularly since the figures are not backed up with any definite evidence. The report says that there is no firm evidence to show that this vaccine's use causes more distress than benefit to the community as a whole.

OHE suggests that in view of the publicly voiced doubts as to the safety of pertussis vaccination priority should be given to a relatively swift action designed to restore confidence before other forms of immunisation are affected. An obvious option would be the temporary withdrawal of routine pertussis vaccine by the NHS, although this is not a recommendation of OHE. Recently the Department of Health and Social Security decided to continue pertussis vaccination while data necessary to make a critical review could be collected. The Institute of Biology endorsed this view when they submitted their views to the Royal Commission on Civil Liability and Compensation for Personal Injury.

Although it is unlikely that any major changes in vaccine policy will be made in the next few years the proposal to withdraw routine BCG vaccination is under discussion.

● AN idea for using the energy in the waves of the sea as a source of safe non-polluting power, developed by Dr Stephen Salter of Edinburgh University has attracted a Department of Trade grant of £60,000. As described by Dr Salter in an article in *Nature's* Energy Review earlier this year (*Nature*, 249, 720; 1974) his system incorporates large steel vanes which harness the rolling motion of the waves, more efficiently than the up and down movement utilised by previous systems.

Apart from the Department of Trade, the Central Electricity Generating Board and a leading British concrete fabricator, Cementation Ltd., are also interested.

Also, Sir Christopher Cockerell, the inventor of the hovercraft, is a partner in a newly formed company, Wavepower Ltd, which is interested in the commercial application development of wavepower.

Astronautics in Amsterdam

from Angela Croome

Dr Kraft A. Ehrlicke belongs to the same generation of German-born space engineers and prophets as Werner von Braun and similarly helped to lay the foundations of the American space programme. It is not therefore too surprising that he retains an almost apocalyptic faith in man-in-space as bringer of solar energy (to supplement dwindling Earth resources) and grower of super crystals. That there are no limits to growth for life on this planet—only changes in pace and direction—was the theme of his keynote address to the 25th International Astronautical Congress, the annual rendezvous of the leading figures in the field, in Amsterdam last week. He urged growth and expansion in astronautics as a way of obtaining additional resources and accelerating the imperatives of international cooperation.

The note was not really in harmony, however, with the main tenor of the meeting. Programme scientists and project leaders today must have hard-headed arguments in justification of even low-cost automatic satellites. An all-day symposium on cost reduction in space operations is now a regular feature of the Congress, in fact. Dr Ehrlicke's industrial suburbs in orbit were not included in the programme.

The principal way of reducing the costs of space operations, of course, is to share them. The degree to which NASA has embraced this policy was heavily underlined at the Amsterdam gathering. Detente has certainly eased the way for American-Soviet space cooperation (where only a few years ago the 'space race' was an extension of the cold war) but the sight of Tom Stafford, captain of the American crew for next summer's Apollo-Soyuz rendezvous and docking project, and Alexei Leonov, the Russian crew leader giving a joint briefing on progress without interpreters gave credibility to the plan that has hitherto been lacking. Officially each country is contributing 50/50 to development, the 5 joint experiments, project management, even the command during the 2 days when the two capsules will become one; unofficially one learns that the exchange of information is all 'one way' and in particular the American space medicine experts responsible for crew fitness are unhappy at the lack of Soviet data.

There is only one American-Soviet flight scheduled at present and nothing definitely planned beyond. Next spring the two sets of senior negotiators (which includes the President of the Academy of Sciences on the Russian side) will consider possible further joint

ventures, and Soviet participation in the reusable Shuttle programme for the 80s has been mentioned. Meantime Europe through ESRO is firmly committed to designing and building the Spacelab element of the shuttle programme which will substantially share the burden of the civilian sector of the programme with NASA. What is now being equally stressed is the virtue of having an integral European contribution to some of the promising planetary programmes of the next 10 years. A system of multiple spacecraft to collect and recover some soil samples from the surface of Mars would be substantially aided by having, for example, Europe build and operate one of the Mars descent vehicles. Similar arguments apply for a 1978 Venus probe in two parts and ESRO is studying a Jupiter mission for the 80s which would be on a joint contribution basis.

A note of caution was struck in the law sessions. The problems of liability in the case of a failure or an accident where several nationals are taking part in a common spacecraft will be formidable and there is no really adequate international court to decide possible space disputes.

Maltese conference cross over Caracas

from Wendy Barnaby, Malta

"It is", said Lord Ritchie-Calder, "the biggest smash-and-grab since the European powers at the Berlin Conference of 1885 carved up Black Africa." And his view of the Caracas United Nations Law of the Sea Conference was echoed by many of his fellow participants at Pacem in Maribus. The fifth meeting of this group, which can claim to be the most influential non-governmental organisation considering matters relating to the oceans, was held in Malta from September 9–13. Attracted to this discussion on the results of the Caracas proceedings were 64 people from 18 nations, the World Bank, the United Nations Environment Programme, the Commonwealth Secretariat and a number of non-governmental organisations. The statement which summarised the meeting's discussion was highly critical of Law of the Sea developments.

It was generally thought that the concept of a twelve nautical mile territorial sea plus a 200 nautical mile exclusive economic zone would eventually be accepted by the U.N. conference, possibly at its next session at Geneva in March 1975. Dr. Arvid Pardo, the original initiator of the U.N. Conference, argued that these concepts will not provide the necessary precision on the scope of national jurisdiction because rules determining the drawing

of baselines, from which each zone will be measured, have not been agreed upon. Nor are they likely to be. Neither has agreement been reached on the relationship of the continental shelf to the economic zone. What will happen to those parts of the shelf which extend beyond 200 miles from the baseline?

As present technology makes exercise by states of their rights to explore and exploit the resources in the economic zone easy to the depth of 200 metres, it would be possible to imagine those states with extensive continental shelves taking advantage of the imprecision of baselines to push their economic zones out to the limits of their shelves. The inequalities this would entail seem too blatant to be borne.

This question of the inequalities built into the proposed system let loose another shower of criticism, this time from Dr. Sidney Holt, the Director of the International Ocean Institute in Malta. He pointed out that although the underdeveloped countries may benefit in the short term from the economic zone concept (on the argument that, even if it will make the rich richer, it will at least give the poor more than they have at the moment), there is no guarantee that their long-term benefit will be great. This is because the potential yield from the living resources within the economic zone is limited to double the present catch, while the potential of living resources much further out is one or two magnitudes greater. This mid-water life, which includes pelagic squid and the shrimp-like krill of the Antarctic ocean, needs special technology to harvest it. It is a reasonable bet that the developed countries will perfect this before the underdeveloped ones. If the latter are not to miss out in the future they should be pressing for international arrangements to enable co-operation with the developed countries in the utilisation of these resources.

A more immediate deficiency in the Caracas machinery was the inconsistency in terms of reference between the Conference's three committees. The second and third committees dealt with the ocean space including the water column, but the first committee was limited to the seabed. The result is that "the authority" which each committee was trying to set up within its own terms of reference will have ambiguous jurisdiction. Clearly this ought to be sorted out quickly at Geneva. And, just as clearly, the Conference should adopt the solution favoured by Pacem in Maribus: jurisdiction that will cover the ocean not just the seabed. The seabed cannot be dissociated from the water column in the sort of issues that will face the world community in its attempt to draw up a law of the seas.

ACCORDING to a remark often attributed to Mark Twain, "everybody talks about the weather, but nobody does anything about it". In the United States, where the federal government spends about \$20 million a year on weather modification research (the actual amount depends on how you define the enterprise) and where the Department of Defense has recently admitted conducting a massive cloud seeding programme in South east Asia, Twain's remark is demonstrably untrue. Nevertheless, nine separate reports in as many years have all said that nobody is doing enough.

The latest in this series, a study conducted by the General Accounting Office (GAO), is no exception for it suggests that weather modification research is financially undernourished, badly coordinated and poorly focused. Such complaints have, of course, been made before for virtually every branch of science but in this case they merit some attention since for one thing the GAO has no axe to grind, and for another they are couched in language which, for the GAO, is uncharacteristically blunt.

A science born in 1946 when Vincent Schaefer and Irving Langmuir seeded rainclouds with pellets of solid carbon dioxide, weather modification moved briskly into commercial application—as much as 10% of the United States was under commercial cloud seeding operations in the 1950s—but it is still full of uncertainties. Even the Pentagon's rain-making activities in Vietnam, for example, are generally regarded as having had only a marginal effect on rainfall there, and most weather modification experiments conducted in the United States have had mixed results.

Nevertheless, a number of organisations including the National Academy of Sciences and a Presidential advisory committee have argued during the past decade that a variety of weather modification techniques show sufficient promise for the federal government to initiate a centrally directed, adequately funded research programme.

But weather modification activities are now spread among some seven federal agencies, and the GAO has found that "an effective overall national weather modification program has not been established". Attempts to coordinate weather modification research, GAO continues, "have been somewhat disappointing", and the fragmentation of effort "has made it extremely difficult for agencies to conduct effective field research".

The GAO identified some \$17.4 million in the federal budget for the 1974 fiscal year (which ended on June 30) for weather modification experiments,

a level which falls dismally short of all the recommendations during the past decade. The National Academy of Sciences, for example, recommended as long ago as 1966 that \$30 million a year could be wisely spent.

But the GAO's chief complaint (and, indeed, that of everybody else who has reported on the matter) is that no agency has been given the power to coordinate and manage an overall weather modification programme.

As an example, GAO singles out the National Hail Research Experiment, a \$16.5 million project begun in 1972 which is designed to test various



methods for eliminating or mitigating the effects of hail storms. Six different departments and agencies are taking part, with the National Science Foundation in a leading role.

Although GAO is quick to point out that the experiment has already yielded some promising results, it lists a number of cases in which agencies failed to meet their obligations, presumably because they accorded the project a low priority. The Department of Commerce, for example, failed to furnish radars and aircraft for the experiment in the first year, the Department of Defense did not supply helicopters as planned, and the National Science Foundation had to put up funds for a part of the project carried out by the Department of Agriculture.

Such problems, GAO reckons, would not have arisen if a national research programme were established and a single federal agency were given responsibility for implementing it.

● THE supplementary agenda of the current session of the United Nations contains a resolution, proposed by the Soviet Union (in a letter of August 7, 1974), urging "The prohibition of action to influence the environment and climate for military and other purposes incompatible with the maintenance of international security, human wellbeing, and health." Not surprisingly, this resolution is receiving much favourable publicity in the Soviet press. While one must applaud the laudable sentiments of this resolution, a certain doubt nevertheless arises; since the Soviet Union is one of the chief exponents of climatic manipulation (for peaceful purposes, be it understood), is there not perhaps the danger that such projects, however beneficial to the country concerned, may result in the deprivation of others, that one nation's plenitude may result in another nation's deprivation.

Apart from grandiose schemes for climatic manipulation in the distant future, such as their longstanding scheme to divert southwards those rivers which at present discharge uselessly into the Arctic Ocean and the creation of artificial glaciers in the deserts of Soviet Central Asia, alarming scheme are proposed in an imaginative survey *Life in the Twenty-First Century*, compiled in 1957. These include the damming of the Bering Straits and the pumping of warm water from the Pacific to the Arctic Ocean, so as to change the climate of Kamchatka, North-East Siberia and the Arctic Basin. Similarly, it is proposed to join Sakhalin Island to the Soviet mainland, and, by pumping warm water from the Kuro-Siwa current through the dam into the Tartar Strait, to warm the Sea of Okhotsk. Naturally, in this panegyric, undesirable side-effects are minimised—there is no more than a joking reference to the Japanese skiing enthusiasts who no longer (in AD 2007) have any snow.

At present, the principal efforts of the Soviet Union are aimed at what *Pravda* calls "hail-prevention" — in reality, hail-making. Research in this field was begun in the late 1960's at the Institute of Alpine Geophysics at Nalchik in the North Caucasus. Potential hail clouds are observed by radar, and then seeded by lead iodide from a rocket fired into the cloud's centre. Although the method appears to be reasonably successful—provided that the hail cloud is spotted in time—it does result in heavy hail falls in surrounding areas. By 1971, the technique was considered sufficiently reliable to form part of the Soviet aid programmes to Argentina, the Lebanon, Hungary and Yugoslavia.

EPA firm on pesticides ban

from Colin Norman, Washington

RUSSELL TRAIN, Administrator of the Environmental Protection Agency, last week held firm on his decision to halt production of the widely used pesticides aldrin and dieldrin, pending the outcome of public hearings on charges that the chemicals may be carcinogenic to man.

The decision is an attempt to establish an important concept in the regulation of environmental chemicals—that possibly dangerous substances should be removed from the market until they are found to be safe, rather than being kept in use until they are definitely found to be hazardous.

That concept is already well established in the regulation of food additives in the United States, thanks to a legal requirement that chemicals cannot be added to food if they are found to increase the incidence of cancer in test animals, but attempts to apply it to the regulation of other environmental chemicals have so far met with mixed

success. Moves against aldrin and dieldrin were made by the EPA as long ago as 1971, when the agency issued an order to cancel the pesticides' registration—which was tantamount to banning their use. That order was based on findings that the pesticides increase the incidence of liver tumours in mice when fed to them in relatively high doses. But the Shell Chemical Corporation, sole manufacturers of aldrin and dieldrin, appealed against the order, and the appeal has been making glacial progress through the courts and EPA's administrative machinery ever since.

Meanwhile, evidence has accumulated that aldrin and dieldrin raise a variety of tumours in at least five strains of mice and possibly rats as well. Their residues can be found in the tissues of virtually every man, woman and child in the United States, and a survey conducted last year by the Food and Drug Administration found the pesticides in 83% of all dairy products, 88% of garden fruits, and 96% of meat, poultry and fish.

On the basis of that evidence, EPA privately asked Shell to stop producing aldrin and dieldrin, at least until the

appeals process on the cancellation order has been exhausted. But Shell refused, and announced that it would produce about 10 million pounds of the pesticides this Autumn for use in 1975, knowing full well that if EPA's cancellation order is upheld next year it would be virtually impossible to call in the pesticides which had already been sold. Train therefore exercised emergency authority in July to order Shell to stop making the pesticides. Shell appealed against that ruling, however, and demanded a public hearing on the matter. The hearing took place in August and early September before Administrative Law Judge Herbert L. Perlman, who upheld Train's order on September 20, as a result of which Train last week refused to reconsider his decision. Shell officials immediately announced that they will take their case to the federal courts.

There is no dispute over the fact that aldrin and dieldrin increase the incidence of liver tumours in mice—in fact, Shell representatives even conceded that point during the public hearings—but there is a dispute over the relevance of that fact for humans.

According to Dr Don Stevenson, Director of Shell's Tunstall Laboratory, for example, five separate criteria must be met before there is sufficient evidence of human carcinogenicity. There must be a higher incidence of tumours in exposed animals, tumours should develop in more than one species, there is adequate proof that the development of the tumours is related to the test compound, the animal has proved to be an adequate model for extrapolating to man, and that there is proof of at least one incidence of cancer in man related to the test compound.

As Train pointed out last week, such criteria would be impossible to meet since cancer in man often takes several decades to develop after the initial exposure. Both Perlman and Train therefore concluded that there is already sufficient evidence of carcinogenicity in mice to suggest that aldrin and dieldrin pose a high risk to man.

Meanwhile, the appeals process for EPA's original cancellation order against the pesticides grinds on. Public hearings have been dragging on since August 1973, and according to Train, they will continue into the "indefinite future". It is worth noting, however, that although the production ban in theory does not prejudice the cancellation issue, Perlman is sitting as hearing examiner for the cancellation hearings as well as for the hearings into the production ban. His 109-page opinion on EPA's production ban represents such a severe indictment of the pesticides that it seems unlikely that he will ultimately give them a clean bill of health.

AFTER the good news about Drumbuie, the bad news about Kishorn. The Secretary of State for Scotland, Mr William Ross, has approved an application by the Howard/Doris partnership to build concrete gravity-type oil production platforms at the Loch (pictured). On the face of it the Kishorn site seems to satisfy none of Mr Ross's criteria for a suitable site; that it must have access to existing facilities, that it can draw on existing labour sources and that it can make use of existing infrastructure.

By giving this decision Mr Ross has brought down on himself a hail of criticism, not only from those who successfully opposed the development at Drumbuie, but also from those who felt that Drumbuie would be the lesser of two evils. As the site is even more remote than Drumbuie from existing rail and road services, all materials and supplies are to be shipped across the loch from the nearest rail link, which will involve an additional development at this point.

But giving his reasons for the decision, Mr Ross emphasised that the very remoteness of the site was a point in its favour. Also, the site will employ a smaller workforce than that proposed at Drumbuie. Obviously a major factor in the differing decisions is the National Trust land involved at Drumbuie, and the fact that far fewer local residents were likely to be affected.



But what must worry many people is the fear that this is only the beginning. This decision has been made despite the recent publication of the Scottish Development Department's Coastal Planning guidelines which designate the West Highlands coast as a conservation zone. Mowlem/Taylor Woodrow who lost the site at Drumbuie, are unlikely to rest under this decision and will no doubt look for a site nearby. As necessary infrastructure is developed, the economic advantages of allowing more sites to use it will no doubt be cited.

A few crumbs of comfort may be gathered in the appendices to the Secretary of State's memorandum. Twelve years from now, the site must be cleared completely of all buildings, and restored to its original condition, with replanting if necessary. Optimists can book their holiday now.

news and views

Genetic engineering with viruses

A SIGNIFICANT development in the field of genetic engineering is described in an article by Drs Noreen and Ken Murray in this week's issue of *Nature* (page 476). They have been able to isolate a mutant of bacteriophage λ which can replace the normally used plasmid in experiments designed to construct new hybrid DNA molecules.

These experiments are very simple in principle and (in brief) involve mixing together in a test tube the donor DNA molecule (the gene to be studied), the receptor (the plasmid) and a 'DNA cutting' enzyme, the restriction enzyme *EcoRI*. The cutting enzyme breaks the circular double stranded plasmid at a single point converting it into a linear molecule. But it does more than this. The enzyme cuts DNA slightly asymmetrically leaving four nucleotides projecting at each end. These 'sticky ends' can pair up by forming Watson-Crick base pairs with similar sticky ends formed on the donor DNA molecule by the cutting enzyme. Thus a linear hybrid plasmid is formed which can circularise. To stabilise the new hybrid plasmid another enzyme, DNA ligase (a 'joining' enzyme), is added to reform the covalent bonds broken by the restriction enzyme. The success of such experiments, which have now been carried out using staphylococcal DNA, *Xenopus laevis* ribosomal genes and *Drosophila* DNA as donor, depends on there being a single site for the restriction enzyme on the plasmid. The problem in phage λ is that there are five sites.

The Murrrays' work involves a brilliant series of genetic and biochemical manipulations to construct a mutant phage λ with one or two sites for the restriction enzyme *EcoRI*. Then, in subsequent experiments, restriction fragments

of these various mutant phage λ s were joined together *in vitro* to form hybrid molecules with a deletion in a non-essential part of the phage. These new molecules were isolated as biologically active phage λ after infection of *Escherichia coli*. By this means the single-site mutant was converted to a bacteriophage with a gap in it leaving room for an incoming piece of DNA. The authors were able to insert the genes specifying the biosynthesis of tryptophan in *E. coli* (the *trp D* and *E* genes) into this receptor phage λ . They imply that they hope to extend this work to study the expression of the *trp* genes, for which phage λ is an ideal host (and has significant advantages over a plasmid host), as well as to study the insertion of DNA from higher organisms.

Many readers of *Nature* will know that there is some controversy among scientists about the ethics of performing genetic engineering experiments (see *Nature*, **250**, 279; 1974). Part of the concern is about the nature of the particular *E. coli* plasmid used in these experiments which confers on the *E. coli* resistance to the antibiotic tetracycline. Clearly, if one wishes to introduce foreign, and possibly hazardous DNA, it is advantageous to use microorganisms susceptible to antibiotics. Dr K. Murray, in a recent lecture to the Biochemical Society, has pointed out that phage λ is much safer than the plasmid. It is sensitive to antibiotics and only infects some strains of *E. coli*. There is also evidence that the laboratory strains for which λ is infective do not establish themselves in the human gut. It is thus improbable that hybrid molecules (of whatever type) could be pathogenic.

G. G. BROWNLEE

Some myths about heritability and IQ

THINGS have gone too far. Estimates of the heritability of IQ reported in *Nature* have assumed a distinctly bimodal distribution. First, Eaves and Jinks¹ decried my data on the heritability of scholastic aptitudes² as a failure to detect the high heritability of IQ. In a sample of urban school-age twins, I found that black and economically disadvantaged children from both races seemed to have lower heritabilities for verbal and quantitative aptitudes than white and middle-class children. In some cases heritabilities were not calculable, because the correlations of opposite-sex twins were slightly larger than those of same-sex twins. Now, Schwartz and Schwartz³ proclaim zero heritability for IQ as the obvious conclusion to be drawn from the same study and from Kamin's iconoclastic efforts (Invited Address, Eastern Psychological Association, Washington DC, March 1973). I beg to differ with both extremes and to argue against any simple conclusions on the issue.

The notion that there is a single answer to the question "What is the heritability of IQ?" is patently false. Heritability estimates vary according to what skills are measured as intelligence, by how they are tested, by the age at which abilities are measured, and by the genetic and environmental composition of the population tested. To claim that there is a single estimate for the genetic contribution to human

abilities is to deny, first, half a century of evidence on differential abilities. Some aspects of intelligence, such as vocabulary, have consistently higher heritability estimates than, say, numerical reasoning. Even if one concluded, with Burt, that general intelligence (*g*) is more important than specific abilities, different tests of *g* will still yield different heritability estimates⁴.

Differences in heritability estimates across abilities and tests are exceeded only by differences across age groups and populations. Bayley Infant Intelligence Test scores for the first two years have been found to have generally lower heritabilities than later scores. Even early heritabilities vary enormously: from 0.2 to 0.4 for siblings (McCall, American Psychological Association, Honolulu, September 1972), 0.75 for some twins (Nichols and Broman, preliminary report of the Collaborative Perinatal Study, 1973), and 0.3 for other twins⁵. Across populations, Nichols⁷ reported a significantly lower correlation for black than white siblings on the Stanford-Binet test at age 4. Another twin study (Scarr-Salapatek, Katz, and Barker, *Black and White Twins*, in preparation) found lower heritabilities for adolescent black and white twin pairs on each of four tests of cognitive abilities and for the first principal component from the four tests. These data are presented in Table 1. Not only are

Table 1 Heritability estimates for four tests of cognitive abilities in two adolescent twin populations

Test	Black h^{*2} ($N=160$ pairs)	White h^2 ($N=211$ pairs)
Raven Standard Progressive Matrices	0.59	0.87
Peabody Picture Vocabulary Test	0.28	0.49
Columbia Test of Mental Maturity	0.42	0.58
Revised Test of Figural Memory	0.61	0.71
First Principal Component	0.48	0.63

* $h^2 = 2(r_{1MZ} - r_{1DZ})$, where r_{1MZ} is the intraclass correlation for monozygotic twins and r_{1DZ} for dizygotic twins.

heritabilities lower for black children on all four measures, the heritabilities are not uniformly 0.80 or 0.00 in either group.

If one observes an array of data from studies of related and unrelated people, the IQ scores of those more closely related generally correlate more highly than those more distantly related. This is undeniable. The view from afar reveals important regularities in the results. If the proponents of a uniformly high heritability would look more closely, however, at the constellation of human differences, they would find important inconsistencies as well.

A recent reanalysis of data on the heritability of Stanford-Binet IQ scores questions the very high heritability estimate that Eaves and Jinks¹ propose. Jencks² reanalysed US family correlations and obtained heritability estimates between 0.45 and 0.60, far below the 0.8 claimed by Burt³, Jensen¹⁰, Eaves and Jinks¹, and Herrnstein¹¹. If Burt's data are included, the heritability estimates rise, in large part because his observed (?) correlations fit genetic expectations so perfectly. As Jensen¹² has recently shown, however, there are serious inconsistencies in Burt's reports. If one relies primarily upon Burt's data, one could be grossly misled as to the proper range of heritability estimates.

A reanalysis of Jencks's reanalysis¹³ suggests that the best fit to the US data is a heritability of about 0.68. But Eaves and Jinks ignore Jencks's major point: that the data from parent-child, twin, sibling, and adopted child pairs do not fit any simple model! There are sufficiently serious disagreements that forcing the data into a biometrical model is to risk a nonsense solution—however mathematically elegant.

In addition, twin data on which the proponents of high heritability rely have been shown to overestimate heritability values¹⁴, presumably because twin environments, particularly those of monozygotic twins, have less variability (pre- and post-natally) than the environments of ordinary siblings. The variance between monozygotic co-twins is further reduced by genotype-environment covariances and interactions. There has not yet been a serious test of the magnitude of covariance and interaction effects on human intelligence. Although Jinks and Fulker¹⁵ suggest a test for interaction (the correlation of half the sum and half the difference between IQ scores of separated monozygotic twins), their barely significant results are based on very small samples.

Further evidence on the exaggeration of heritability values comes from Morton¹⁶. He revived Wright's¹⁷ reanalysis of Burks's¹⁸ adoption study. Burks's faulty path analysis model yielded a heritability estimate for general IQ (in white California adoptees) of 0.75 to 0.80. Wright's more adequate path model resulted in a heritability of 0.50 from the same data. Morton's additional calculations for sibs and foster sibs gave a heritability solution of 0.52.

The idea of zero heritability strikes most geneticists and biologists as odd (Fuller, presidential address, Behavior Genetics Association, Minneapolis, June 1974). How can there be any biological characteristic for which genetic variability makes no contribution to phenotypic variability? As Morton¹⁶ remarked, "... the experience of biometrical genetics [is] that a trait heritable at its extremes has never been found to have zero heritability within the normal

range" (page 257). Certainly IQ is affected by many single gene and chromosomal anomalies. There are four possibilities to explain a finding of zero heritability: first, the measurement of the phenotype is invalid or unreliable; second, there is no genetic variability underlying the phenotype; third, all genotypes are functionally equivalent in producing the phenotype; or fourth, other effects overwhelm genetic variability. The first two possibilities for the heritability of human abilities can be dismissed out of hand. The third is what Schwartz and Schwartz⁷ want to argue. There is simply too much evidence to the contrary, however, from studies other than those that they and Kamin would dismiss. Though every human family study may be criticised on one or more methodological grounds, their flaws are varied and non-overlapping. The weight of evidence suggests that genetically related persons are more similar intellectually than unrelated persons, whether they are reared together or apart. The microscopic examination of research flaws has blinded some critics of heritability to the obvious pattern of results.

The fourth is what Henderson¹⁹ and I^{20,21} argued for some populations. Other effects can include rearing conditions, growth patterns at a particular age, the skills tested, and many unspecified conditions that will affect sources of variability. The negligible and low heritabilities reported for aptitude scores among disadvantaged, school-aged twins in Philadelphia² were a function of the environments sampled, the tests, the populations, and possibly the age group. The point is that genetic differences did not seem to contribute much to variability in aptitude scores in those populations. Different tests, ages, populations, and cohorts may well yield other results. Since these tests are very widely used to make life decisions for children, however, their low heritabilities have considerable implications.

There is no single answer to the question "What is the heritability of IQ?" because it is a pseudo-question. Heritability estimates range between 0.00 and 0.9 in reported research, but most values are in a middle range. An accurate view of genetic contributions of differences in IQ cannot be obtained from the proponents of extreme positions. In fact, the best view of human variability can be obtained by getting off the political platform and out there to study families. With the notable exception of Arthur Jensen, not many advocates of high or low heritability are adding to our store of knowledge about human intelligence.

SANDRA SCARR-SALAPATEK

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⁴ Vandenberg, S. G., in *Intelligence: Genetic and Environmental Influences* (edit. by Cancro, R.), 182 (Grune and Stratton, New York, 1971).

⁵ Vandenberg, S. G., in *Genetics, Environment, and Behavior* (edit. by Ehrman, L., Omenn, G. S., and Caspari, E.), 276 (Academic Press, New York, 1972).

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¹¹ Herrnstein, R., *IQ in the Meritocracy* (Atlantic, Little, Brown, Boston, 1973).

¹² Jensen, A. R., *Behav. Genet.*, **4**, 1 (1974).

¹³ Eaves, L. J., and Jinks, J. L., *Nature*, **248**, 287 (1974).

¹⁴ Layzer, D., *Science*, **183**, 1259 (1974).

¹⁵ Jinks, J. L., and Fulker, D. W., *Psychol. Bull.*, **72**, 311 (1970).

¹⁶ Morton, N. E., in *Genetics, Environment, and Behavior* (edit. by Ehrman, L., Omenn, G. S., and Caspari, E.), 247 (Academic Press, New York, 1972).

¹⁷ Wright, S., *J. Am. statist. Ass. Suppl.*, **26**, 155 (1931).

¹⁸ Burks, B. S., *Yb. natn. Soc. Stud. Educ.*, **27**, 219 (1928).

¹⁹ Henderson, N., *J. comp. physiol. Psychol.*, **3**, 505 (1970).

²⁰ Scarr-Salapatek, S., *Science*, **178**, 229 (1972).

²¹ Scarr-Salapatek, S., *Science*, **182**, 1044 (1973).

Origin of metalliferous sediments

FOR reasons which are largely historical and political rather than scientific, the first specific phenomenon likely to come to mind at any general mention of ocean mineralisation is that of manganese nodules. No doubt there is some justification for this emphasis, for in spite of the many unsolved problems relating to both their collection and processing, nodules will almost certainly be the first deep sea minerals to be exploited commercially. But nodules are not the whole story of marine mineralisation, nor, as Bullard (*Endeavour*, 33, 80; 1974) has pointed out, will they necessarily prove to be the most important part of it in the long run. Nodules are attractive because they are discrete conglomerations of a few metals in a particularly concentrated form, but in both economic and scientific terms they are likely to be rivalled increasingly by sediments containing unusually high proportions of similar elements.

The story of the discovery of metal-rich sediments begins in 1948 when Swedish workers on the research vessel *Albatross* discovered a region of abnormally high temperature in the Red Sea just north of latitude 21° N. About 15 years later, more detailed investigations of water temperature and salinity were carried out in the same region by teams from the United States, Germany and Britain; and as a result of the discoveries made then, a further study of both water and underlying sediments was undertaken in 1966 by the research vessel *Chain* from the Woods Hole Oceanographic Institution. These expeditions found three deep basins containing hot water with a salinity about seven times greater than that of normal Red Sea water. The largest of these 'brine pools' was the Atlantis II Deep (about 15 km long by 5 km wide), although it became clear that this is not the deepest of the brine-filled basins when Backer and Schoell (*Nature phys. Sci.*, 240, 153; 1972) reported the discovery of 13 more brine pools by the German ships *Wando River* and *Valdivio*.

But if the water in the Red Sea deeps is remarkable, equally so are the sediments beneath them. For whereas the normal sediments in the central trough of the Red Sea are light-coloured marls, those in the brine pools were found to comprise yellow, red, brown and blue layers enriched in such metals as iron, manganese, copper and zinc. The enrichment seems to be high but quite variable. In 1969 Bischoff (in *Hot Brines and Recent Heavy Metal Deposits in the Red Sea*, Springer-Verlag, 1969)

said that the high grade sediments in the Atlantis II Deep contain an average of 3.4% Zn and 1.3% Cu after removal of salt and water. But Tooms (*Endeavour*, 31, 113; 1972) has quoted a more recent survey by a commercial company which led to average estimates over a much wider area of 6% Zn and "several per cent" Cu. Even more recently, Backer and Schoell have claimed that some facies contain 50–65% iron, that the average manganese concentration in the Chain Deep is 30–40% and that some sulphide layers in the Atlantis II Deep contain 20% zinc, although they admit that "continuously changing facies conditions" reduce the average base metal content to a few per cent.

But although quoted metal concentrations differ, there is complete agreement that enrichment has taken place; and this inevitably raises the question of how and under what circumstances it occurred. The Red Sea is widely believed to be an embryo ocean, and the deeps containing hot brine are closely associated with the central trough. The connection between metallogenesis and seafloor spreading is thus unmistakable—but is metal enrichment a feature only of spreading in its early stages or does it occur whenever and wherever spreading takes place? The thick salt deposits covering a large part of the Red Sea depression must be the ultimate source of the salt in the brine pools, and the brine pools are closely associated with the enriched sediments beneath. Does this mean that metal enrichment only takes place in relatively closed oceans conducive to the formation of salt deposits by repeated evaporation, or is the presence of abundant salt in itself an unnecessary condition?

These questions cannot yet be properly answered. But there is now abundant evidence for the existence of metal-rich sediments on the East Pacific rise and other ocean ridges. Such sediments were apparently known to Revelle (*Publs Carnegie Instn.*, 556; 1944) thirty years ago, although modern studies can probably be said to date from the geochemical work of Bostrom and Peterson (*Econ. Geol.*, 61, 1258; 1966) following the discovery of the critical part played by ocean ridges in global tectonics. In this and numerous subsequent reports, Bostrom and his colleagues and others have shown that metal enrichment (but not apparently to economic concentrations) does indeed take place along ridges in late stages of spreading; and Deep Sea Drilling Project results have indicated that such sediments are not

uncommon at the base of sedimentary columns throughout the ocean basins.

The general circumstances in which metalliferous sediments are likely to form are thus at least partly understood; but precisely how they form is still an unsolved problem. Nevertheless, it is becoming increasingly clear that hydrothermal processes are involved in some way. In the case of the Red Sea, for example, it may be presumed that the salt in the brine comes from the existing salt deposits, but the water must have acquired its temperature from the upwelling magma at the central rift. It is then reasonable to suppose that, during contact with the magma, the water also acquired metallic components which later precipitated out as metallic compounds to form the observed sedimentary ores.

In fact, water may well penetrate to substantial depths (possibly several kilometres) in the new oceanic crust at ridge axes, thus leaching a considerable volume of rock. Both Corliss (*J. geophys. Res.*, 76, 8128; 1971) and Spooner and Fyfe (*Contr. Mineral. Petrol.*, 42, 287; 1973) have proposed models in which sedimentary metal enrichment could occur by this mechanism; and Lister (*Geophys. J.*, 26, 515; 1972) has used deep hydrothermal circulation to explain the unexpectedly low average heat flow at ridge crests and the high scatter of heat flow values at ridge crests and flanks. Piper (*Earth planet. Sci. Lett.*, 19, 75; 1973) and many others have offered geochemical evidence; and on page 473 of this issue of *Nature*, Anderson and Halunen present combined geophysical and geochemical data which give further support. As Lister (*Eos*, 55, 740; 1974) has rightly suggested, what is required now is a commitment to test the hydrothermal circulation hypothesis more directly by deep sea borehole measurements.

PETER J. SMITH

Cell culture pharmacology

from Mary Dawson

THE action of drugs on cultured vertebrate cells was the subject of the autumn meeting of the British Society for Cell Biology, held at the University of Strathclyde, Glasgow, on September 19 and 20, 1974. Most of the experiments described had one of four main objects—to increase knowledge of the cells being studied by using known compounds as tools, to elucidate the mode of action of drugs where this was unknown or partly known, or to design *in vitro* tests which would either provide knowledge about new drugs under development or would form a basis

for choosing the best drug from a range of possibilities for any one patient. Of the papers presented more had been engendered by cancer research than by any other single disease area. This is a fair representation of cellular pharmacology work since it is logical and practicable both to study the effects of compounds on normally and abnormally proliferating cells and to use compounds to study differences between such cells.

The Searle Lecture was delivered by L. J. Tolmach (Washington University) who spoke of his group's work on the enhancement of radiation effects on proliferating cells by various drugs used separately or together. He emphasised, as did several subsequent speakers, the relative ease of carrying out statistically satisfactory numbers of replicate experiments in cell culture, and of automating additions and removals of drugs. Drug-radiation interaction was discussed also by A. H. W. Nias (Belvidere Hospital, Glasgow) and papers on specific groups of compounds were presented by A. M. Creighton (Imperial Cancer Research Fund) and B. J. Phillips (Chester Beatty Research Institute).

The papers of R. I. Freshney (Beatson Institute, Glasgow) and J. A. Dickson (Royal Victoria Infirmary, Newcastle-upon-Tyne) dealt with the design of cell culture tests for measuring human tumour sensitivity to drugs, and the place of such tests in cancer therapy. They described the uses and limitations of such tests, and factors influencing the results, pointing out that a laboratory LD₅₀ end point still meant 50% of surviving cells too.

A further group of four papers was on the subject of cardiac glycosides and electrophysiology experiments. These were by J. F. Lamb (University of St Andrews) and W. F. Dryden *et al.* (University of Strathclyde). There were two papers on anaesthetics and related compounds by L. J. Erkel (Göteborg) and A. G. Macdonald (Aberdeen). The latter paper led to comment on the validity of using non-mammalian material to investigate clinical problems, as did the paper of D. Brown, M. Balls and J. Pryor (University of East Anglia) who chose amphibian cell cultures for their relative functional longevity. The symposium title had been deliberately widened from "human" to "vertebrate" cells to generate discussion on the possibility of errors due to species difference on extrapolating from various types of *in vitro* tests to clinical situations. There was indeed much discussion on choice of culture material.

The action of parathyroid hormone on explanted bone rudiments was discussed by P. J. Gaillard (University of Leiden) in relation to hyperparathyroid

conditions, and P. J. Hornsby and M. J. O'Hare (Chester Beatty Institute) described functional activity in cultured adrenal cells. The extraordinary biological properties of the cytochalasins were discussed by S. B. Carter (Imperial Chemical Industries, Macclesfield) who indicated their value in examining cellular processes as did M. de Brabander (Janssen Laboratories, Beerse).

Further areas where cell culture study was relevant to finding information about the compounds or the cells were reported in the papers on prostaglandins by M. Adolphe (Institute of Pharmacology, Paris), on *in vitro* antibody production by G. Harris (Kennedy Institute, London), on bacitracin by M. Dawson (University of Strathclyde) and on colchicine and microtubules by D. Wheatley (University of Aberdeen).

Mystery molecule in Japan

from Sydney Shall

THE Japanese have taken poly(ADP-ribose) to their heart. Since much of the progress in this area of molecular biology comes from Japanese laboratories, this is understandable. The occasion of the first Oji International Seminar at Tomakomai, Hokkaido, was used by Professors Osamu Hayaishi (University of Kyoto) and Takashi Sugimura (University of Tokyo) to organise the third international meeting on poly(ADP-ribose) and the ADP-ribosylation of proteins (September 10-12). This very young field has progressed steadily over the last five years, but without success so far in two major respects: a simple method for measuring poly(ADP-ribose) in intact cells, and unequivocal evidence of its physiological function are both lacking.

This unusual chromosomal polymer has a composition almost identical to that of poly(A) but does not have a nucleic acid structure. It is made by a chromosomal enzyme for which NAD⁺ is the sole substrate. NAD⁺ metabolism in cancer cells is unusual and poly(ADP-ribose) is thought to have some role in DNA replication and cell proliferation.

The highlights of the meeting included progress in the purification (5,000 times) of the poly(ADP-ribose) polymerase by Ueda and Hayaishi (University of Kyoto). The partially purified enzyme still requires both DNA and histones and makes short chains. The enzymes that degrade the polymer have been isolated and purified by L. Burzio (Rockefeller University, New York) from testis, and by M. Tanaka, M. Miwa and T. Sugimura (University

of Tokyo) from liver, thymus, *Physarum polycephalum* and from tobacco cells in culture.

There was considerable discussion following the paper by H. Hilz (University of Hamburg) as to whether the polymer is covalently attached to chromosomal protein. Hilz demonstrated that at least some of the polymer is covalently bonded to protein.

The most interesting report was the description by T. Kanai and T. Sugimura (Tokyo) of the preparation of antibodies to poly(ADP-ribose). They showed that with these antibodies a radioimmunoassay for poly(ADP-ribose) will be possible. The antibodies seem to be specific and do not cross-react with DNA, RNA or poly(A). In addition, they have detected anti-poly(ADP-ribose) antibodies in the sera of human patients with the autoimmune diseases, especially in systemic lupus erythematosus. H. Hilz has estimated the amount of poly(ADP-ribose) in intact animals by an extremely carefully controlled, isotope dilution technique. Three important conclusions emerge: the polymer exists in intact cells, the amount of polymer was estimated in adult rat liver to be 5.25 ± 0.09 nmol of ADP-ribose residues per mg DNA; no striking difference in amount was found between adult rat liver, neonatal rat liver and Ehrlich ascites carcinoma cells. But in isolated nuclei there is a substantial difference in the content of mono(ADP-ribose) residues between adult and neonatal liver.

Other examples of ADP-ribosylation of proteins are very well authenticated. The modification and alteration of DNA-dependent RNA polymerase by ADP-ribosylation after T4 phage infection of *Escherichia coli* was clearly demonstrated by W. Zillig (Max-Planck-Institut, Munich) and C. G. Goff (Medical Research Council, Cambridge). ADP-ribosylation of mammalian ribosomal elongation factor II by diphtheria toxin was dissected by C. Edson, K. Ueda and O. Hayaishi (University of Kyoto) and E. S. Maxwell (National Institutes of Health, Bethesda). Both laboratories have so far failed to identify the ribosyl-protein bond in modified elongation factor. But Maxwell showed the presence of an unusual amino acid in unmodified elongation factor II to which the ADP-ribose was apparently attached by the diphtheria toxin. Finally, K. Yoshihara (University of Nara, Japan) adduced evidence that the recently described chromatin-bound Ca²⁺, Mg²⁺ endonuclease is itself ADP-ribosylated.

The mysteries of poly(ADP-ribose) are steadily yielding to systematic attack. The elucidation of its function will radically alter our picture of the biology of the cell nucleus.

Fish out of phase

from Marian Dawkins

ANIMALS often need to distinguish between stimuli which arise from their environment (which includes other animals) and those which are caused by some action of the animal itself. An apparent movement of an object or a sound, for example, may require a completely different response depending on whether or not the animal has produced that movement or that sound by its own behaviour, even though the pattern of stimulation reaching the animal's sense organs may be extremely similar in the two cases.

Although the ways in which animals make this distinction are many and varied, a recently described example in a species of electric fish by Scheich (*Science*, **185**, 365; 1974) is of particular interest as it illustrates how the distinction can be made even in a situation where the animal must distinguish between two similar and simultaneous wave forms.

Scheich studied a species of fish, *Eigenmannia*, in which the electric organ has a regular wave-like discharge, enabling the fish to locate objects by detecting disturbances in the resulting field. When two *Eigenmannia* which are discharging their electric organs at similar frequencies come close enough together, they both unerringly adjust their frequencies in opposite directions so that their frequencies are no longer similar and the danger of interference to their object-detecting systems is avoided.

Conveniently for experimental study, a fish will also alter its frequency if stimulated through an electrical dipole in its tank at a frequency near its own. Within wide limits of absolute frequency, the fish will correctly alter the frequency difference between its own discharge and that of the applied stimulus to maintain a difference. In order to do this the fish must be able to detect how near its own frequency is to that of the applied stimulus and to have information on whether it is lower or higher.

A possible, although cumbersome way of doing this would be to have an array of frequency-tuned filters and for the fish to respond whenever two filters near enough in frequency were active together, provided it had some way of telling which frequency filter was responding to its own discharge. In fact, as Scheich demonstrates, the fish's solution is much simpler and his theory accounts more satisfactorily for the fish's ability to recognise the sign of the frequency difference whatever the fish's own frequency within that range.

The analysis is done by sensitivity to the beating of the two signals in

the electric field—that is, the coming into and out of phase of the two waves of slightly different frequency. The frequency with which the two waves come into phase with one another (like the frequency of beat notes with two sound waves) is the difference between the two frequencies and will be independent of absolute frequency.

If the electric organs discharged sinusoidally there would be ambiguity as to which signal belonged to which fish, but the discharge is not sinusoidal: the wave shape is rich in harmonics and is clipped in one polarity. By being sensitive to the peculiarities of beat patterns produced by the interaction of two such oddly shaped waves, the fish can tell whether the interference is above or below it in frequency as well as how near in frequency. By ingenious experiments in which the fish's own output was converted artificially into a sine wave, Scheich found that under these circumstances, the fish was completely unable to distinguish the sign of the frequency difference between its own output and a sinusoidal applied stimulus. Only when its own output was allowed to resemble more closely the normal discharge was its normal behaviour restored. Scheich also found neurones in the fish's mid-brain which responded to differences in frequency in such a way that they could directly provide the input into the electric organ.

Thus the fish is able to obtain information about how near its own frequency another fish is discharging and whether the other is above it or below it in frequency, not by analysing constituent frequencies, but rather by beat analysis in the time domain. It is interesting that a similar mechanism is postulated by some theories of hearing.

RNA molecules in initiation of protein biosynthesis

from Alan E. Smith

TEN years ago the main interest in protein biosynthesis was the establishment of the genetic code by translating synthetic polynucleotides of known composition or sequence in bacterial cell-free systems. Then it seemed reasonable to suppose that any ribosome can attach to almost any RNA molecule and translate it into polypeptide. This view is now known to be totally naive. The initiation of protein synthesis is a highly complex interaction between a ribosome, the initiator tRNA, and the specific ribosome-binding site on the mRNA. Several protein factors which also play a part in this process have

various enzymatic functions: dissociating the ribosome into subunits, binding the initiator tRNA and binding the appropriate mRNA molecule. It now seems that in addition to the protein initiation factors, there are RNA molecules involved.

For some time, Heywood and his colleagues have been studying the specificity of mRNA translation in heterologous cell-free systems and have concluded that a specific mRNA can only be translated in a heterologous ribosome if a specific homologous initiation factor (designated IF₃) is present. Thus the synthesis of myosin on, say, erythroblast ribosomes requires initiation factors obtained from muscle ribosomes (*Biochemistry*, **11**, 2061–2066; 1972). When they further fractionated red muscle initiation factors required for the synthesis of myoglobin and myosin in a reticulocyte cell-free system, Heywood, Kennedy and Bester (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 2428–2431; 1974) found that two activities are present: one specifically stimulates myoglobin synthesis and the other stimulates myosin synthesis. In addition to these two proteins, an RNA molecule was isolated from the IF₃ preparation and this seems to inhibit the initiation and translation of heterologous mRNA molecules. Bogdanovsky, Herman and Schapira (*Biochem. biophys. Res. Commun.*, **54**, 25–32; 1973) have previously shown that a similar RNA molecule of molecular weight 11,000 is present in preparations of initiation factors from rabbit reticulocytes and that the RNA is required for the synthesis of globin *in vitro*.

The presence of a small RNA initiation factor may corroborate the observations of Reichmann and Penman. They found that the base analogue 5-azacytidine inhibits protein synthesis in HeLa cells with a half life of 90 minutes (*Biochim. biophys. Acta*, **324**, 282–289; 1973). The inhibition seemed to occur at the initiation of protein synthesis and was suppressed by actinomycin D. Earlier studies had established that the half life of mammalian mRNA is very long compared with the response to aza-cytidine, and this suggested that an RNA molecule other than mRNA or rRNA, which is rapidly metabolised *in vivo*, is involved in the initiation of protein synthesis. The authors then showed (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 2678–2682; 1973) that the putative RNA species accumulates in cells subjected to inhibition of protein synthesis by, for example, cycloheximide or starvation of an essential amino acid, and cell-free systems prepared from such cells show a greatly enhanced ability to initiate new polypeptide chains.

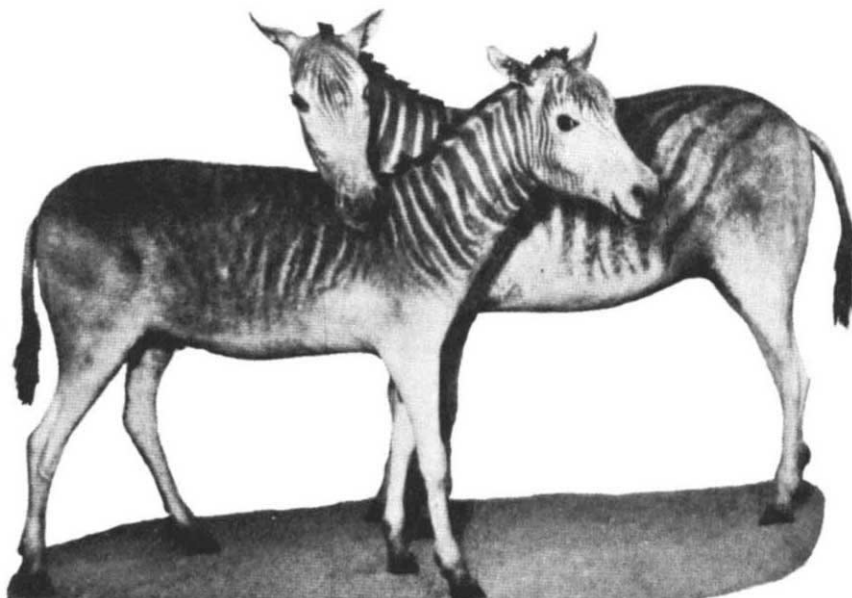
The evidence available to date indicates that the RNA species involved

in initiation is not rRNA. Probably, therefore, the phenomenon described above is not related to the small 2S RNA species recently reported in *Drosophila* ribosomal RNA (B. R. Jordan, *FEBS Lett.*, **44**, 39–42; 1974) since this seems to be a cleavage product of 26S rRNA maturation. It is possible, however, that the mode of action of the small stimulatory RNA in eukaryotes resembles that proposed for the 3'-OH end of 16S RNA in bacteria. Shine and Dalgarno (*Proc. natn. Acad. Sci. U.S.A.*, **17**, 1342–1346; 1974) have sequenced the terminal portion of the rRNA from several bacteria and found that certain oligonucleotides near to the 3'-OH are complementary to GGAGGU—a sequence common to many of the mRNA ribosome binding sites that have so far been examined. Perhaps such a structure selects the correct site of mRNA and positions it on the ribosome. It is conceivable that the small RNA species described above has a similar function in eukaryotes.

The new RNA species not only has initiation factor activity but can also discriminate between different mRNAs. This means that a change in the specificity of initiation can be effected either by a protein (IF₃) factor or by an RNA species, and adds a further dimension to experiments in which changes in the specificity of initiation factors are being examined, for example, at different stages in development (Sierra, Meier and Ochoa, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2693–2697; 1974). The presence of small RNA molecules in initiation factor preparations from uninfected cells may also suggest a function for those ribosome-associated RNA molecules that are found during viral infection but apparently do not function as mRNAs. For example during reovirus and adenovirus infections, small viral RNA molecules have been detected in excess of those required to code for viral proteins—these RNAs could alter the specificity of translation on host cell ribosomes, either by shutting off host cell synthesis or preferentially favouring viral protein synthesis.

Double-stranded RNA also has a profound effect on the initiation of protein synthesis. This was first described by Ehrenfeld and Hunt (*Proc. natn. Acad. Sci. U.S.A.*, **68**, 1075–1078; 1971) who were searching for the inhibitory agent responsible for shutting off host protein synthesis in poliovirus-infected cells. The effect on initiation was detected in rabbit reticulocyte lysates and was remarkable because of the small amounts (about 0.1 ng ml⁻¹) of ds RNA required. Celma and Ehrenfeld (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 2440–2444; 1974) have now reassessed the possible role of ds RNA in the shut-off phenomenon by studying the

Shades of meaning



Male quagga in Naturhistorisches Museum, Mainz on left; Burchell's zebra (type of *E. quagga paucistratus*) on right.

THE quagga, *Equus quagga quagga*, no longer roams the Cape Colony having succumbed to the hunter's gun in the nineteenth century. The last quagga died in the Amsterdam Zoo in 1883, but it is thought that this one had outlived by several years the wild quaggas in South Africa.

The striping and coloration of this close relative of the modern zebra have long been contentious subjects. There has also been controversy about whether the quagga is a true subspecies or just a geographical variant of Burchell's zebra, *E. q. burchelli*. In an attempt to sort out the confusion, Rau of the South African Museum in Cape Town, has scoured Europe for quagga remains and he now presents his revised list of what he considers true quagga material (*Ann. S. Afr. Mus.*, **65**, 41–87; 1974).

In the past, the quagga has been described variously as having black or dark stripes on a brown or fawn base with white or whitish legs and ventral surface; or being dark brown, chestnut or fawn coloured with white or whitish stripes on the antero-dorsal area of the animal. Authors writing this century have favoured a light striped animal.

After studying preserved quaggas in 27 European museums Rau concludes that there is no sharp distinction between the quagga, *E. quagga quagga* and *E. q. burchelli*. It just seems to represent, with many variations, the extreme southern limit of the trend seen in the plains zebra group: that is, in the south, the white darkens towards brown and the black lightens to a brown. This process gradually culminates in the disappearance of the stripes.

initiation of protein synthesis in cell-free extracts of uninfected and poliovirus-infected HeLa cells. Host and viral protein synthesis are equally sensitive to ds RNA and much higher concentrations (about 5 µg ml⁻¹) of RNA are required to inhibit synthesis in the HeLa cell-free system. Since such concentrations of inhibitor are not present until late in poliovirus infection Celma and Ehrenfeld suggest that ds RNA does not act directly to inhibit host cell protein synthesis. Such a conclusion is supported by experiments reported in the same issue of *Proc. natn. Acad. Sci. U.S.A.* (**71**, 2549–2553; 1974) by Thach and colleagues, who have determined by electron microscopy the structure of the purified replicative intermediate of encephalomyocarditis virus (a picornavirus closely related to

polio). These authors conclude that before deproteinisation the replicating RNA contains very little ds RNA, thus the inhibitory agent detected in polio-infected cells may be an artefact caused by extraction of the viral RNA.

But three further considerations should be weighed in favour of ds RNA as the inhibitory agent before it is completely rejected. First, cell-free extracts from HeLa cells are never very active compared with whole cells: second, disrupted cells may contain an RNase III type activity which destroys much of the added ds RNA but is without such activity *in vivo* and finally even though full length ds RNA may not be present in infected cells presumably the area immediately surrounding the polymerase molecule is in a double stranded form; the effect of ds RNA in

reticulocyte lysates, which lack RNase III, is so potent that this small region of poliovirus ds RNA might be sufficient to produce the inhibitory effects seen *in vivo*.

The mechanism of action of ds RNA in reticulocyte lysates is the subject of another recent paper in *Proc. natn. Acad. Sci. U.S.A.* Benzard and London (71, 2863–2866; 1974) have shown that the inhibitory effect can be prevented or reversed by addition of the initiation factor M₃. Preparations of initiation factors M₁ and M₂ (which are responsible for the binding of initiator tRNA) and ribosome dissociation factor are ineffective. These observations are in agreement with similar experiments by Kaempfer and Kaufman (*Proc. natn. Acad. Sci. U.S.A.*, 70, 1222–1226; 1973) who showed that ds RNA inactivates reticulocyte initiation factor IF₃, but both reports conflict with those of Darnbrough, Hunt and Jackson (*Biochem. biophys. Res. Commun.*, 48, 1556–1564; 1972) who found that ds RNA inhibits the formation of the complex between 40S ribosomal subunits and initiator tRNA. At present it is difficult to resolve this apparent contradiction. But it seems that very small amounts of ds RNA inhibit initiation by sequestering a catalytic site which normally recognises some structural feature in another RNA molecule. Such a structure could be present in mRNA, rRNA, initiator tRNA or perhaps even the small RNA molecule discussed earlier.

Archaeopteryx flies again

from Barry Cox

Archaeopteryx is probably the best example of an evolutionary intermediate between two major groups of animal. It is normally accepted that it is not far removed from the arboreal reptilian stock from which birds evolved. Though its skeleton is not greatly modified for flight, the elongated feathers of *Archaeopteryx* strongly suggest that flight of some kind had already evolved.

Ostrom (*Q. Rev. Biol.*, 49, 27; 1974) has now questioned the idea that bird flight evolved in an arboreal environment. He starts from the observation that *Archaeopteryx* and the smaller cursorial dinosaurs such as *Ornitholestes* show so many characteristics in common that it would be surprising if their habits of life were totally different. He also states that the terminal phalanges of the foot of *Archaeopteryx* are relatively straight and robust, like those of cursorial birds but unlike those of perching birds, which are strongly curved.

It has long been noted that, surprisingly, the manus of *Archaeopteryx* has three long, unfused clawed fingers, and these have been interpreted as adaptations to climbing in trees. Ostrom shows that the hand, and the whole pectoral skeleton, of *Archaeopteryx* are very similar to those of such dinosaurs as *Ornitholestes*, and that the claws were long, curved and pointed—more suitable, he thinks, to a prey-grasping function than to climbing. He also notes that neither the pectoral skeleton nor the long many-feathered tail of *Archaeopteryx* suggests that it could have been an active flier.

Ostrom's view is that *Archaeopteryx* was a bipedal fast-running form which lived on "relatively small animals, most probably large insects, and perhaps small lizards and mammals". He believes that feathers evolved to aid in thermal insulation, and that their elaboration on the forelimb was originally to increase its efficiency as a prey-catching appendage, the two arms "becoming a trapping device or net with which to corral or surround small prey so that they could be more easily grasped in the mouth or hand". True flight, Ostrom believes, evolved from flapping, leaping attacks on such prey.

Long-established theories are often found to be based on deductions which are today either discarded or less certain, and it is usually worthwhile to attempt a fundamental reappraisal. Ostrom's work seems certain to provoke further discussion and criticism—both of the orthodox theory and of his own views. Walker (*Nature*, 237, 257; 1972) has already suggested that birds are more closely related to crocodiles than to theropod dinosaurs, but both Gingerich (*Nature*, 243, 70; 1973) and Bakker and Galton (*Nature*, 248, 168; 1974) have supported the link with dinosaurs—the latter even to the extent of including birds in a new class Dinosauria.

Some aspects of Ostrom's theories seem somewhat unlikely, at least at first glance. It is always heartening to the palaeontologist to be able to see similar adaptations in use today, but there are none such for *Archaeopteryx*'s supposed insect-snaring spread of feathers. These would also have been of little use (and possibly a hindrance) in catching the small lizards and mammals that Ostrom proposes as alternative prey. His suggestion that the long, feathered tail was a braking mechanism seems equally unconvincing. Ostrom may well be right in suggesting that the ancestors of *Archaeopteryx* evolved bipedality as ground-running forms, like some modern lizards (in which bipedal and quadrupedal locomotion are not fundamentally different), and that they only evolved the extensive spread of feathers as a gliding adaptation after they had

become arboreal forms. Nevertheless, the adoption of a dramatically different method of locomotion (flight) might well have followed from colonisation of a very different habitat, such as trees. The imperfection of *Archaeopteryx* as an arboreal gliding form and its retention of a grasping hand might then merely indicate a relatively recent adoption of this way of life, rather than disproving it.

Other interested parties are girding their loins for the fray, and it will be interesting to see whether *Archaeopteryx* ends up with its feet on the ground or its head in the air.

Agricultural waste to supplement oil

from D. A. H. Taylor

IN 1949 Dupont opened a plant at Niagara for the production of nylon intermediates from furfural. This has long been closed down, as it could not compete with petrochemical routes, but it may now be time to reconsider the position of furfural in the light of the present oil situation.

Furfural is obtained by acidic hydrolysis of pentosans, contained in agricultural waste. Almost any plant material may be used, including straw and sawdust, with an average yield of 5–10%. Present sources include oat hulls, maize cobs, and bagasse, which is the residue after extracting sucrose from sugar cane. The potential supplies are large: it has been calculated that 2 million tons of furfural could be produced per annum in the United States from maize cobs alone (Dunlop and Peters, *The Furans*, 283, Reinhold, New York; 1953); the potential yield from sugar cane is roughly equal to that of sucrose, currently about 50 million tons yr⁻¹. This may be compared with a world production in 1970 of approximately 40 million tons of primary petrochemicals (Imhausen, *Chem. Ind.*, 1559; 1970.)

It is possible to make practically any petrochemicals one wishes from furfural, at a price. As the sources of furfural are usually of slight alternative value, the main expenses in production are plant construction, operating costs and handling and collection charges for the raw material. Its present uses are mainly as a solvent and in the synthesis of resins used to manufacture grinding wheels and brake linings. A major use is the production of resin for impregnating the glass fibre mats wrapped around steel pipelines to prevent corrosion, and currently the demand in connection with the construction of Arctic pipelines has raised the price of furfural delivered in Europe

from less than £200 to more than £650 a ton. By comparison, the current price of naphtha feedstock, used for the production of petrochemicals, is of the order of £60 per ton. At this level, oil prices have still far to go before furfural could compete as a fuel for heating or power plant use. Furfural itself does not seem to have attractive properties for use as a fuel for internal combustion engines, but chemical derivatives are more promising.

Furfural derivatives usually require hydrogenation to be useful chemically which is unfortunate because hydrogen is rich in energy and hence expensive. The Dupont nylon process first decarbonylated furfural to furan by catalytic treatment, and then hydrogenated the furan to give tetrahydrofuran. This was then cleaved with hydrogen chloride to form 1, 4-dichlorobutane, which with sodium cyanide gave adiponitrile, a conventional intermediate for nylon 6/6.

Tetrahydrofuran is a valuable intermediate in other ways; one fifth of German wartime butadiene was produced by vapour phase dehydrogenation of tetrahydrofuran over a phosphate catalyst (Dunlop and Peters, *The Furans*, page 730). Although no longer used, this process would become viable again if alternative supplies of butadiene became more expensive, and gives access to a wide range of products, including the synthetic rubbers. Tetrahydrofuran has also potential as a fuel for internal combustion engines. Although its properties in this respect have not been investigated in detail it seems promising either alone or together with hydrocarbons, with which it is readily miscible.

The tetrahydrofuran process involves the loss of one carbon atom from the furfural molecule, and an alternative route which avoids this is to hydrogenate furfural to tetrahydrofurfuryl alcohol. This on heating in the gas phase gives 2, 3-dihydropyran, which may be hydrogenated to tetrahydropyran. This is also a potential fuel and chemical intermediate; analogously to tetrahydrofuran it can be converted to 1, 3-pentadiene, or to pimelonitrile and hence to nylons of the 7/7 series. Extensive development work has been done on these and other possible routes for utilisation of furfural in the chemical industry.

Hydrogenation of butadiene or pentadiene will give the saturated butane or pentane, which are in the main stream of petrochemical practice. Butane is currently used to manufacture motor fuel.

Thus increased furfural production may help to solve at least some of the present and future problems produced by the increased price and declining supply of crude oil.

Protein in two environments

from R. Colin Hughes

THE classical concept of amphipathic membrane proteins defined by Winzler, Morawiecki, Singer and their colleagues is a polypeptide containing separated domains of peptide sequences of polar or non-polar character. This seems to occur in the major erythrocyte membrane glycoprotein, a molecule in which oligosaccharide units are attached exclusively to the amino terminal half of the polypeptide. The carboxyl terminal half of the chain contains an amino acid sequence which is unusually hydrophobic and seems to be embedded in the lipid bilayer of the membrane. As Singer has pointed out, however, an amphipathic protein may consist of a linear polypeptide of more mixed character that only when folded produces the two distinct segments, one highly polar and the other highly hydrophobic, required of an integral membrane protein. Sequence analyses of more membrane proteins are obviously of great interest, and information of their three-dimensional structure cannot be too long delayed.

In the meantime, however, the field is open for speculation. In the June issue of the *Proceedings of the National Academy of Sciences* (71, 2396-2400; 1974) Inouye produces a particularly ingenious model for one membrane protein—the lipoprotein of the outer membrane of the *Escherichia coli* envelope.

The bacterial envelope consists of two distinct membranes: the inner or cytoplasmic membrane and the outer membrane, which are separated by a thin layer of peptidoglycan. The outer membrane contains a large amount of lipopolysaccharide and a unique lipoprotein, thoroughly characterised by Braun and his colleagues. The lipoprotein just penetrates the lipid bilayer of the outer membrane to peep out both at the bacterial external surface and at the inner surface towards the peptidoglycan, to which the lipoprotein is in part covalently linked. The sequence data of Braun show that the lipoprotein consists of 58 amino acid residues. An unusual amino acid, glycylcysteine [S-(propane-2', 3'-diol) 3-thio-2 amino-propanic acid] is N-terminal and linkage to the peptidoglycan involves the C-terminal lysine residue, which is linked to the carboxyl group of meso-diamino pimelic acid.

The lipoprotein has a high α -helical content and Inouye puts forward two possible arrangements in which the α helix is twisted to allow the hydrophobic amino acid residues to face in one direction whereas the hydrophilic

residues face oppositely. These arrangements are of course compatible with an integral membrane protein, provided that the twisted helices are aggregated to form a superstructure in which the hydrophilic faces are in contact and the outer surface of the structure is hydrophobic. This is satisfied either by a cylindrical arrangement of six helices or by six α -helical coils arranged in a superhelix or a coiled coil, with the hydrophobic residues of each α helix pointing outwards. The height of the superhelical assembly will be about 76 Å, sufficient to penetrate through the 75 Å thick outer membrane with hydrophobic interaction between the surface of the assembly and the lipid bilayer of the outer membrane. The lipoprotein molecules may be stabilised further in the bilayer through lipid-lipid interactions involving the three fatty acids attached to the N-terminal residue (two by ester linkage and one by amide linkage). Since one or more of the individual α -helical polypeptides of the assembly may be attached covalently to the underlying peptidoglycan, the lipoprotein assembly is constrained and free diffusional motion within the outer membrane presumably is not allowed.

The function of the bacterial lipoprotein is unknown. Inouye points out an intriguing aspect of his model, however, in that the superstructure of assembled helices spanning the membrane could provide hydrophilic pores through the outer bacterial membrane. Two general classes of molecular mechanisms for protein-mediated transport involving pores may be postulated. A penetrating membrane protein could be itself porous. Alternatively, the pores could be formed by an assembly of protein molecules: the proteins are porogenic rather than porous. In the case of the bacterial lipoprotein, Inouye calculates a hydrophilic pore size of from 12.5 Å to 35.8 Å depending on an assembly of six or twelve α helices. The number of lipoprotein molecules per bacterium is about 7.5×10^5 , and accordingly the calculated total number of channels per cell varies from 1.25×10^5 to 0.63×10^5 . That is, about 35% to 45% of the total bacterial cell surface is occupied by the assemblies. These hydrophilic channels could accommodate the passive diffusion through the outer bacterial membrane of almost all materials required for growth, provided of course that diffusion of these substances is also allowed through the other barriers of the peptidoglycan layer and the cytoplasmic membrane.

articles

Search for pulsed X rays from 3U0833-45 (Vela pulsar)

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A search for pulsed X rays from 3U0833-45 has been carried out. The Uhuru error box for this source ($\sim 8' \times 24'$) contains the Vela pulsar. We obtain an upper limit of $\sim 1/3$ the intensity of 3U0833-45 for periodic pulsing with the 89-ms period of the Vela pulsar. Other evidence for X-ray emission from the Vela pulsar is reviewed.

SOME 100 radio pulsars have been discovered. The spin periods range from 33 ms (NP0532) to 3.75 s (NP0525). Only two of these pulsars have been reported to emit X radiation: the Crab pulsar (NP0532) and the Vela pulsar (PSR0833-45). In the case of NP0532, the pulsed X-ray emission is rather intense, making it the 25th brightest X-ray source in the sky. Its high energy emission covers the range from 1 keV to greater than 1 GeV (refs 1-3).

The Vela pulsar is the next most likely emitter of X rays because it has the next highest spin frequency, ~ 89 ms. There have been various reports of X-ray emission, and lack of emission, from it. In the 2 to 10 keV range, a source (3U0833-45) has been detected in the vicinity of the Vela pulsar by Uhuru⁴. The error box for this source is $\sim 8' \times 24'$ and contains the Vela pulsar; however, the time resolution of Uhuru is insufficient to reveal a possible 89 ms pulsing. The diffuse Vela supernova remnant has not been detected in the 2 to 10 keV energy range.

A marginally significant detection of X-ray pulsing from the Vela pulsar in the energy range 23 to 80 keV has been reported⁵, but with a period 155 ms different from the radio period. In the same energy range the MIT balloon group found no pulsing (2σ confidence) at the same level of intensity⁶. In soft X rays, 0.5 to 1.5 keV, the AS&E group found pulsing with the same period as in the radio and with an intensity of $\sim 2\%$ of the total X-ray intensity of the Vela supernova remnant⁷. The same group also reported a non-pulsed component about twice as intense as the pulsed component⁷. These latter results are inconsistent with a recent pulsing limit set by the CalTech group⁸ ($< 0.3\%$ of the intensity of the Vela supernova remnant) and the detection by the Copernicus satellite of only a much weaker source within $6'$ of the Vela pulsar⁹. The time resolution of the Copernicus X-ray instrumentation during the Vela observation was inadequate to detect 89 ms pulsing. The experimental results are summarised in Fig. 1.

Our data

The present experiment, which features 1 ms time resolution, was directed toward a search for periodic (89 ms) pulsed X-ray emission from 3U0833-45 in the energy range 1.5 to 10 keV. An earlier search in the same energy range¹⁰ yielded an upper limit substantially above the intensity of 3U0833-45. The present observations were made from an Aerobee 170 sounding rocket

launched from Woomera (Australia) at 1800h, November 9, 1973 (UT). The payload instrumentation consisted of a bank of 6 beryllium window proportional counters, sensitive in the energy range 1.5 to 10 keV with 900 cm² total open area. During

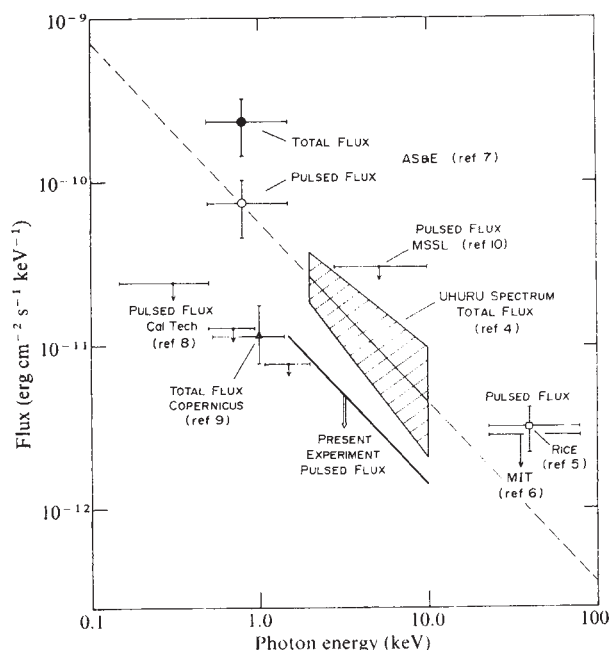


Fig. 1 Summary of X-ray data for the Vela pulsar. The pulsing limits from the present experiment (1.5 to 10 keV) are derived under the assumption of an energy spectrum with a spectral index $\alpha = 1.1$ (same as for 3U0833-45, ref. 4). For different assumed spectra, the limits set by the heavy line in the figure would rotate about a fixed point near 3 keV (5×10^{-12} erg cm⁻² s⁻¹ keV⁻¹) where the detectors are most sensitive.

the final 100 s of the flight, the view axis of the detectors was pointed in an intermediate direction between Vela X-1 (3U0900-40) and the Vela pulsar. Only 70 s of the Vela data were recovered because of a single 30 s loss of the telemetry signal. The instrumentation and the results of the observations of Cir X-1 (observed earlier in the flight) and Vela X-1 have been presented previously¹¹.

The observations in the Vela region yielded about 6,500 signal counts above a background of $\sim 38,000$ counts, due mostly to the isotropic X-ray flux. We estimate that 3U0833-45 contributes about 600 of the 6,500 signal counts. We arrive at this estimate by folding the spectrum of 3U0833-45 (ref. 4: $I(E) = 5.6 \times 10^{-11} E^{-1.1}$ erg cm⁻² s⁻¹ keV⁻¹) with the response function of our detectors. The calculation indicates a total source counting rate for the present experiment of 9 counts s⁻¹. The data were folded modulo the apparent period of PSR0833-45 at the time and place of the rocket launch, $P = 0.0892230$ s

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(private communication from P. Reichley). The resultant histogram (Fig. 2) shows no obvious or statistically significant features. The hatched peak superimposed on the data represents the expected appearance of the light curve if 3U0833-45 were 100% pulsed and had a light curve identical with the radio pulse profile of PSR0833-45 at 2.4 GHz (single pulse ~ 4 ms wide¹²).

Implications for models

So most of the signal from the Uhuru source 3U0833-45 is either pulsed with a wide duty cycle ($\geq 30\%$) or is a steady non-pulsing flux. A steady X-ray flux could be synchrotron radiation from a nebular source surrounding the pulsar although there have been no reports of a compact steady non-thermal radio source¹³. The observed emission cannot be blackbody radiation from the hot neutron star surface since the required temperature, $\sim 10 \times 10^6$ K, is too large for the current age ($\sim 10^4$ yr, ref. 14) of the pulsar. Also, the X-ray flux at the Earth would be almost two orders of magnitude larger than observed, even for a minimum neutron star radius of ~ 5 km and a distance of ~ 1 kpc, which is twice that given in ref. 15.

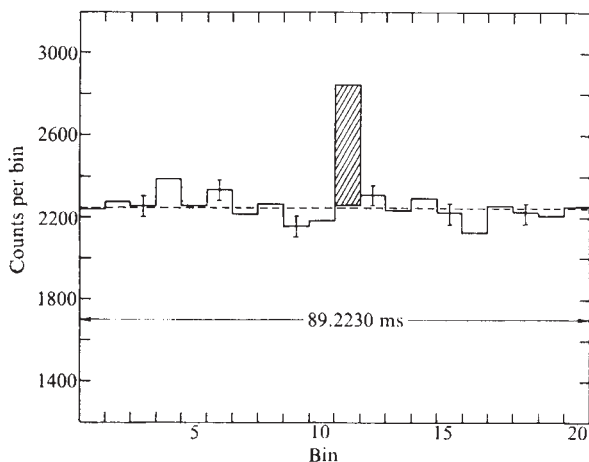


Fig. 2 X-ray data from the Vela region superimposed modulo the apparent period of PSR0833-45. The time reference for zero phase, (start of bin 1) is the launch time: 18 h 00 min 00 s, November 9, 1973 (UT). About 99% of the counts are from the isotropic X-ray background and 3U0900-40. The hatched peak represents the expected light curve if 3U0833-45 were 100% pulsed with a duty cycle similar to that of the radio pulsar. The phase of the expected peak is arbitrary since we were unable to obtain the phase of the radio pulse.

The limit (3σ) for the time averaged pulsed X-ray emission is 3×10^{-11} erg cm⁻² s⁻¹ (1.5 to 10 keV) or 5×10^{-12} erg cm⁻² s⁻¹ keV⁻¹ at 3 keV for an assumed $E^{-1.1}$ energy spectrum and pulse profile similar to that of the radio pulse. In this context, we note that the X-ray and radio pulse shapes for the Crab pulsar are similar^{1,16}. If the width of the X-ray pulses from PSR0833-45 is as broad as 12 ms, then the upper limit to pulsed emission which we can set is about 5×10^{-11} erg cm⁻² s⁻¹. The time averaged X-ray luminosity then becomes

$$L_{\text{Vela}} (1.5 \text{ to } 10 \text{ keV}) \leq 8 \times 10^{32} \text{ erg s}^{-1}$$

for a distance of 500 pc and a 4 ms pulse produced by a broad fan beam. This compares with the X-ray luminosity of the Crab pulsar, for a distance of 2 kpc (refs 1, 17):

$$L_{\text{NP0532}} (1.5 \text{ to } 10 \text{ keV}) = 9 \times 10^{35} \text{ erg s}^{-1}$$

The Crab pulsar therefore radiates at least 1,000 times the power of the Vela pulsar in the 1.5 to 10 keV range. This ratio is still greater than 150 if the Vela pulsar is as far as 1 kpc and the Crab is as close as 1.5 kpc. If the X-ray luminosity of pulsars has a power law dependence on the spin period, P , ($L_x \propto P^{-n}$), then these ratios yield an index n which is probably greater than seven and conservatively greater than five. The value $n = 4$ is

predicted for the total luminosity by the oblique rotator or unipolar inductor models¹⁸⁻²⁰. If one further assumes that a fixed fraction of the rotational energy loss is emitted in the form of pulsed X-rays (ref. 21), then the present result is incompatible with these models. The luminosity dependence on spin period predicted by the "corpuscular model" ($L \propto P^{-7}$, ref. 22) and that predicted for incoherent synchrotron radiation close to the speed-of-light circle ($L \propto P^{-10}$, ref. 23) seem to be consistent with the present X-ray observations. The calculated X-ray luminosity for incoherent radiation from the "electron polar zones" ($L \propto P^{-17/4}$, ref. 24) is inconsistent with the present result both in its dependence on spin period and in its absolute value.

A similar comparison at optical wavelengths has been made previously. The time averaged apparent magnitude of NP0532 is $m_v = 16.5$. No detections of PSR0833-45 have been made down to limiting magnitudes $m_v \sim 24$ (refs 25, 26). This leads to a conservative upper limit to the optical luminosity ratio²⁵:

$$L_{\text{Vela}}/L_{\text{NP0532}} < 2 \times 10^{-4}$$

which is even smaller than in the X-ray region. The index n for the optical case is then greater than ~ 8.5 , but this holds only if there is no severe optical extinction for the Vela pulsar.

Further X-ray observations of 3U0833-45 are required to provide an improved position to confirm the identification with the pulsar and to search for lower levels of pulsed X-ray emission.

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Implications of heat flow for metallogenesis in the Bauer Deep

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The Bauer Deep, in the south-eastern equatorial Pacific, is the only deep ocean basin so far known to be characterised by metalliferous sediments throughout the depositional column. The basin was formed when the eastern Pacific spreading centre jumped from the now fossil Galapagos Rise to the newly formed East Pacific Rise.

The distinctly bimodal distribution of heat flow in the Bauer Deep indicates that precipitation from hydrothermal fluids emanating from the oceanic crust may be the mechanism for metallogenesis in the Bauer Deep. The hydrothermal circulation may have been initiated by the emplacement, approximately ~ 6 Myr BP, of a heat source associated with the ridge jump into that vicinity.

THE existence of metalliferous sediments on the East Pacific Rise and other mid-ocean ridges has been known for some time¹⁻³. Such deposits are a characteristic of basal sediments from Deep Sea Drilling cores throughout the ocean basins (see refs 4-6). The Bauer Deep in the south-eastern Pacific, is the only deep-ocean basin known at this time in which metalliferous sediments have been reported throughout the depositional column. Sayles and Bischoff⁷ reported concentrations of Fe and Mn of up to 18% and 6.5%, respectively, and low concentrations of Al and Ti in sediments containing little or no CaCO₃. They also reported anomalously high concentrations of Cu, Ni, and Zn.

Extensive mapping of the regional extent of the ferruginous sediments has been an integral part of the IDOE Nazca Plate project (G. R. Heath *et al.*, unpublished). Cores from JOIDES, leg 34, site 319 (Fig. 1) have recently shown more than 100 m of metalliferous sediment in the Bauer Deep⁸, with large concentrations of metalliferous components throughout the entire section. Cores from leg 34, holes 320 and 321 (Fig. 1) also revealed basal segments rich in heavy metals, although the upper sections of each were relatively free of anomalous metal concentrations.

Corliss⁹ argued that metallic sediments from the mid-

ocean ridge crest were precipitated from hydrothermal solutions percolating upwards from the basaltic oceanic crust. He argued that metals are mobilised by dissolution in seawater circulating in the crestal provinces of ridges. Williams *et al.*¹⁰, Lister¹¹, and Sclater *et al.*¹² have presented extensive evidence, based upon a bimodal, oscillatory heat-flow pattern, for massive hydrothermal circulation in the crestal province of mid-ocean ridges. The seafloor spreading process itself thus provides the heat source, volcanism, faulting, and fracturing necessary to drive hydrothermal circulation in this environment.

Sediments from the Bauer Deep are chemically similar to sediments from the East Pacific Rise in that both have high transition metal: aluminium ratios¹³⁻¹⁵, with sulphur, uranium, and O₂ isotopic compositions consistent with formation from seawater^{13,14}, strontium isotopic compositions suggesting equilibrium with seawater^{14,15}, rare-earth patterns like that of seawater^{9,13}, and lead isotopic compositions which are similar to mid-ocean ridge tholeiites¹⁵. The mineralogies of the two areas are, however, quite distinct—the ridge crestal metals are found as oxides and the Bauer Deep metals are found as silicates⁷—and the mode of formation is by no means settled.

The origin of these metalliferous sediments and, in general, iron-manganese deposits throughout the oceans is still an unsolved problem. Bonatti *et al.*¹⁶ suggested four different mechanisms of origin. First, hydrogenous, that is, slow precipitation from seawater in oxidised environments. These deposits are found as microparticles dispersed in sediments, nodules and concretions on abyssal plains, and solid pavements on topographic highs. Second, hydrothermal, that is, precipitation from hydrothermal solutions in areas of volcanism and/or high heat flow. These deposits are found along active oceanic ridges and rifts and are associated with submarine volcanism. Third, halmyrolytic, that is, manganese supplied by submarine weathering of basaltic debris. Fourth, diagenetic, that is, precipitation resulting from diagenetic remobilisation of manganese in the sediment column.

There are at least three major hypotheses for the occurrence of metallogenesis in the Bauer Deep. Metals may be precipitated from hydrothermal solutions actively circulating there today¹⁵. Alternatively, they may be hydrogenous and may have nothing whatsoever to do with hydrothermal circulation in the basin. Excessively slow sedimentation rates could produce the large concentrations of metallic components; or some metals may be released into the water column at mid-ocean ridges by hydrothermal circulation and others may be hydrogenously deposited in the Bauer Deep¹³. Little geophysical evidence has so far been brought to bear on this problem.

We present a compilation of heat-flow measurements in the area and draw upon the anomalous nature of the thermal structure of this basin to propose that extensive emanation of hydrothermal fluids¹⁵ can account for both the observed metalliferous sediments and the heat-flow distribution in the Bauer Deep. A qualitative model is presented, associating both with the emplacement of a new heat source into old oceanic crust during the jumping of the major north-south seafloor spreading centre in the eastern Pacific from the Galapagos Rise to the East Pacific Rise.

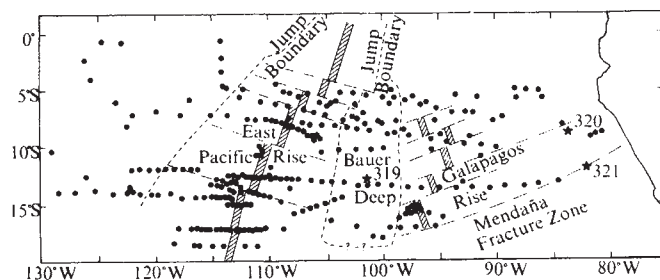


Fig. 1 Physiography of the south-eastern equatorial Pacific Ocean. Solid circles, locations of heat-flow stations plotted in Fig. 2 (sources from refs 20, 27-31, and R. N. Anderson *et al.*, unpublished); hatching, active and inactive spreading centres; light dashed lines, fracture zones; stars, JOIDES, Leg 34, hole locations⁸.

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Physiography

The Bauer Deep is bounded on the west by the East Pacific Rise, on the north by the Galapagos Spreading Centre (both are active seafloor spreading mid-ocean ridges), on the east by the Galapagos Rise, a fossil spreading centre, and extends to the south to at least the Mendaña fracture zone at 16° S on the Nazca Plate (Fig. 1). The basin was formed by the westward jumping, about 6 Myr ago, of the spreading centre from the Galapagos Rise^{17,18}, 800 km to the west to form the East Pacific Rise in crust which was at least 10 Myr old at the time of the jump^{17,19}.

The Bauer Deep has a marked topographic step bordering the basin on the west, and a relatively smooth slope up to the Galapagos Rise on the east (Fig. 2). The elevation offset across this western step indicates an age discontinuity from crust which is about 6 Myr old, and which was generated at the crest of the East Pacific Rise to the crust of the Bauer Deep which is about 10–16 Myr old and which was generated at the Galapagos Rise.

The western boundary of the basin is also marked by extremely rough topography. These ridges are probably not primary topographic features emplaced at the spreading axis, but are either uplifted basement⁸ or extrusive ridges emplaced at the time of the ridge jump. Basement at site 319 (Fig. 1) is extremely fresh compared with other drilled basalts recovered just below basal sediments of the same age (~15 Myr) and the composition of basalts is more alkaline than tholeiites from the ridge crest⁸. Therefore, the basement could be 'off-ridge extrusions' younger than the basal sediments⁸.

Heat flow distribution

Our heat-flow compilation is part of an extensive discussion of the heat-flow distribution on the Nazca Plate (R. N. Anderson *et al.*, unpublished). A heat flow against age plot for the eastern Pacific shows that the Bauer Deep is characterised by high, but variable heat flow (Fig. 2). The variability of heat flow commonly decreases as the average heat flux decreases with age away from mid-ocean ridges²⁰. The mean and standard deviation of heat-flow values from the Bauer Deep are twice as large as values for oceanic

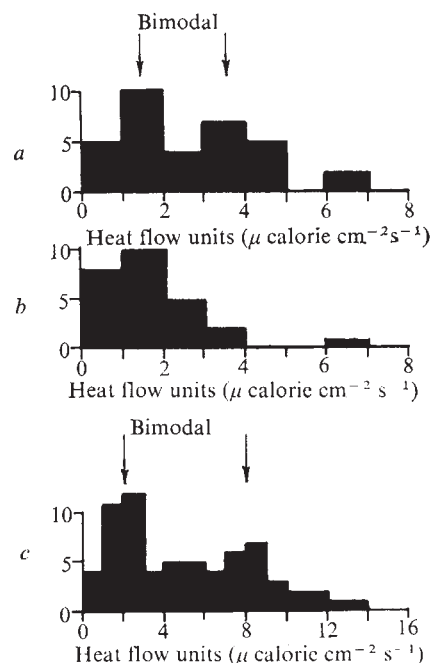


Fig. 3 Histogram of heat-flow values in a, the Bauer Deep— $n=33$ —(crustal ages 10–16 Myr BP, from Fig. 2); b, the eastern Pacific— $n=26$ —(ages 10–16 Myr BP, from Fig. 6 of ref. 20); c, the Galapagos Spreading Centre— $n=67$ —(0–1 Myr BP, from ref. 10).

crust of the same age in other ocean basins (see ref. 20). The observed mean heat flow departs significantly from the heat flux predicted by an empirical relationship between heat flow and age compiled for the entire eastern Pacific²⁰ in one area only—the Bauer Deep (Fig. 2). High heat flow and large scatter in values are evident around the crests of the East Pacific Rise and the Galapagos Rise, as well as in the Bauer Deep. This is a characteristic of mid-ocean ridge heat flow patterns in general (see ref. 10). For crust 10–16 Myr old (the age of the Bauer Deep) the empirical heat flow against age relationship²⁰ was determined from 26 heat flow measurements in areas of the eastern Pacific away from ridge jumps and fossil ridges. These 26 measurements do not show the bimodal heat flow pattern characterised by the Bauer Deep values (Fig. 3). Rather, they show a normal distribution about their mean, of $1.4 \mu\text{calorie cm}^{-2} \text{ s}^{-1}$ ($\sim 60 \text{ mW m}^{-2}$), (Fig. 3). A comparison of crust 10–16 Myr old, east and west of the Galapagos Rise (Fig. 2), again shows the anomalous nature of the thermal structure of the Bauer Deep. Such variability is characteristic of conductive heat flow measurements in areas dominated by convective heat transfer which is associated with hydrothermal circulation^{10–12}. Heat flow values from the Bauer Deep (Fig. 3) show a distinct bimodal distribution characteristic of hydrothermal circulation areas such as the Galapagos Spreading Centre (Fig. 3). The heat-flow pattern at the crest of that mid-ocean ridge shows a 5 km wavelength oscillation of heat flow away from the ridge. Variations from $0.1 \mu\text{calorie cm}^{-2} \text{ s}^{-1}$ (4.1 mW m^{-2}) to $>33 \mu\text{calorie cm}^{-2} \text{ s}^{-1}$ ($>790 \text{ mW m}^{-2}$) were measured over a 4 km distance. This variability, the systematic oscillation, and observed water temperature anomalies which indicate hot spring activity led Williams *et al.*¹⁰ to comment that there was conclusive evidence for widespread circulation of seawater into the oceanic crust on the Galapagos Spreading Centre.

In addition, thermal conductivity (K) measurements in the Bauer Deep made both on recovered cores²¹ and *in situ*²² produced some values 100% greater than those measured in 'typical' deep-ocean sediments. Values of 3.9 and $3.7 \text{ mcalorie cm}^{-1} \text{ s}^{-1} \text{ } ^\circ\text{C}^{-1}$ (1.6 and 1.55 W mK^{-1})

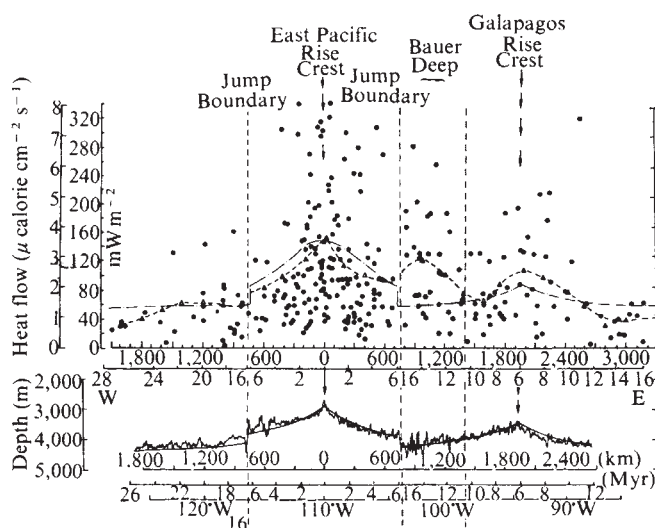


Fig. 2 Top, heat flow plotted against age for the south-eastern equatorial Pacific. Only those measurements shown in Fig. 1 are used for this compilation. Bottom, composite bathymetric profile (from Anderson and Schlater¹⁸) showing representative topographic features in the area. Solid circles, heat-flow values; open triangles, means for 400 km overlapping intervals; solid curves, empirical elevation against age (from ref. 25); — — —, empirical heat flow against age (from ref. 20); dashed line, averaged observed heat flow for the south-east Pacific.

were measured at 14°37.2'S, 104°33.2'W, and 14°53.3'S, 105°15.8'W (R. N. Anderson *et al.*, unpublished). The banding of layers of high conductivity was measured in cores from the Galapagos Rise (A. J. Halunen, jun., unpublished). Thus, the average heat flow in the Bauer depression ($2.65 \mu\text{calorie cm}^{-2} \text{ s}^{-1}$ ($\sim 100 \text{ mW m}^{-2}$) for 33 measurements) may be biased low because most measurements have been determined by assuming conductivities from nearby locales rather than from measurement of sediments collected at the site. The existence of the newly discovered high conductivities in the metalliferous region implies to us that many assumed conductivity values may be as much as 25% too low.

Metallogenesis model

Spooner and Fyfe²⁴ have presented a model for seafloor metamorphism which involves the circulation of seawater through oceanic crust at mid-ocean ridges, chemical exchange with basalt during hydration of the oceanic crust, and subsequent upwelling of hot, hydrothermal solution into the sediment column. They argue that inorganic precipitation from the hot brines can account for heavy metal enrichment found in ophiolitic cherts.

As already mentioned, Bauer Deep sediments have sulphur, uranium, oxygen, and strontium isotopic compositions and rare-earth element patterns consistent with formation from seawater¹³⁻¹⁵, and lead isotopic compositions similar to those of mid-ocean ridge tholeiites⁹. The latter led Dasch *et al.*¹⁵ to conclude that the iron-rich sediments from the Bauer Deep originate as precipitates from hydrothermal solutions injected into sea water. Bischoff and Sayles²⁵ point out, however, that the pH and major ion concentrations of the pore fluids from the core examined by Dasch *et al.*¹⁵ do not differ significantly from the ion concentrations in the overlying bottom water. Sayles and Bischoff⁷ therefore concluded that "at this point, it is not possible to evaluate the role of 'volcanic emanations' in supplying the major components of Bauer Deep sediments". We wish to add the previously unrecognised geophysical constraint of anomalously high and variable heat flow to the accumulating body of data, mostly geochemical, which will ultimately lead to the determination of the mode of origin of metalliferous sediments in the Bauer Deep.

A model can be suggested which can account for both the anomalous thermal structure and the metalliferous deposition in the Bauer Deep. The important distinction to be made is that hydrogenous precipitation of heavy metals cannot cause the anomalous heat-flow distribution in the Bauer Deep. Areas of high heat flow could be caused by hot hydrothermal fluids moving from the basaltic crust into the sedimentary column. The uniformly thin sediment cover in the basin means that such heat would be conducted to the surface in a relatively short time ($<100,000$ yr), (R. N. Anderson *et al.*, unpublished). Low heat flow would follow this initial period of high flux since significant heat would have been removed from the oceanic crust¹². Therefore, hydrothermal circulation can account for the bimodality of observed measurements in the Bauer Deep.

The Bauer Deep is the only area of extensive metalliferous sediments so far discovered away from a mid-ocean ridge crest. It is also characterised by extremely rough topography associated with an age boundary or step marking a jump in spreading centres from the Galapagos Rise to the East Pacific Rise. The mechanics of this jump required the emplacement, 6 Myr ago of new, hot material from the ridge crest into crust at least 10 Myr old (ref. 18) at that time. The introduction of this new heat source into the area may have initiated hydrothermal circulation which had been dormant since the previous thermal event contiguous with the seafloor spreading process. Dehydration reactions driven by the reheating may have released

H₂O for advection to the sediment interface and opened previously clogged cracks to allow further circulation¹². Otherwise, the association between metalliferous sediments adjacent to a major ridge jump and the bimodal distribution of heat flow in the area must be related to different mechanisms and must be coincidental. The association of both on ridge crests seems, however, to argue against coincidence.

Additionally, JOIDES holes 320 and 321 show evidence for a cessation of hydrothermal precipitation approximately 10 Myr BP. Hart *et al.*⁸ concluded that this may be related to the westward jumping of the ridge. They imply that precipitation may be generally related to proximity to the spreading process heat source.

Heat flow may prove to be an adequate exploration tool for the discovery of further metalliferous sediments on the sea floor. The process of ridge jumping is not unique to the Bauer Deep. That also occurred between 25°S and 35°S on the Nazca Plate, for example. This is perhaps an ideal place to prospect for heavy metals.

The IDOE Nazca Plate Project provided important geochemical information on composition and distribution of metalliferous sediments on the Nazca Plate. This work was funded by the National Science Foundation and the Office of Naval Research. J. Greenslate, J. Natland, R. Moberley and V. Vacquier reviewed the manuscript.

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Manipulation of restriction targets in phage λ to form receptor chromosomes for DNA fragments

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Fragments of DNA from derivatives of phage λ having either one or two targets for R.EcoRI have been joined in new combinations to give biologically active phage genomes. These include two classes of deletion mutants; in one, only the DNA between the targets is deleted, but in the second the deletions are more extensive. Other fragments of DNA have been inserted into these phage chromosomes.

RECENT advances in the biochemistry of DNA offer means of creating combinations of genetic material that cannot be achieved by genetic recombination. Biologically active DNA molecules have been reconstituted from separated, sheared halves of bacteriophage λ DNA by the successive action of λ exonuclease and polynucleotide ligase¹ and a derivative of λ DNA has been fused to an animal virus genome². The highly specific restriction endonucleases³⁻⁵ have become powerful new tools for the fragmentation of DNA molecules and the study of the organisation of these fragments within viral genomes^{6,7}. Of special interest in the present context are the enzymes that make breaks a few base pairs apart in opposite strands of duplex DNA to produce fragments with short, complementary, single-stranded projections or cohesive ends⁸⁻¹¹. Such DNA fragments can be joined by annealing the cohesive ends and closing the single-strand breaks with polynucleotide ligase¹².

The first restriction enzyme of this type to be described was isolated from a strain of *Escherichia coli* carrying the RI drug-resistance factor⁹. Some small, circular DNA molecules have only one target^{12,13} for the RI restriction endonuclease, R.EcoRI¹⁴, making them suitable receptors for R.EcoRI frag-

ments of DNA from other sources. Insertion of a fragment of DNA into the single R.EcoRI site of plasmid pSC101 destroyed neither the tetracycline-resistance phenotype nor the ability of the plasmid to replicate, so that the recombinant DNA molecules were able to transform *E. coli*¹⁵. This type of experiment has important potential for cloning, and hence studying, DNA from other sources. Bacteriophage λ , with its extensively studied genetics and biochemistry could be a particularly useful alternative vehicle for applications of this sort. The DNA of phage λ , however, has five targets for R.EcoRI¹⁵, so our first problem was to construct derivatives of λ whose chromosomes have only one target for this enzyme. We have made a series of such phages and shown that their DNA can be broken with R.EcoRI to give fragments which we have joined in new combinations, thus introducing specific deletions. We have also shown that fragments of DNA produced by the action of R.EcoRI can be inserted between two fragments of λ DNA having respectively the right and left ends of the phage DNA. These recombinant DNA molecules were isolated as plaques following transfection of *E. coli*.

Construction of phage with single targets for R.EcoRI

Treatment of phage λ DNA with R.EcoRI gives six fragments of DNA separable by electrophoresis in polyacrylamide or agarose gels¹⁵. These fragments result from breakage of the DNA within the five restriction targets whose positions on the physical map of the λ chromosome have been deduced (Fig. 1)¹⁵ (R. W. Davis, personal communication). We noted that two of these targets, designated *srI1* and *srI2* according to the con-

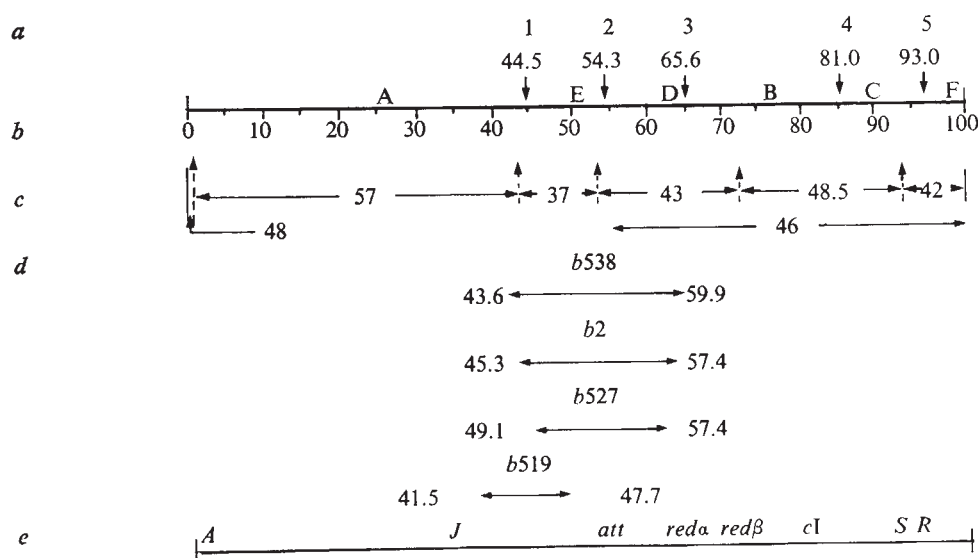


Fig. 1 Some characteristics of the λ chromosome. *a*, Positions of the targets, *srI1*–*5*, recognised by endonuclease R.EcoRI (R. W. Davies, personal communication) and location of the DNA fragments (A–F) (identified in Fig. 2) resulting from breakage of λ DNA by this enzyme. *b*, Unit length (%). *c*, The G + C content (%) of DNA fragments obtained by shearing²². The composition of DNA to the right of *srI2* was calculated from that of the sheared fragments. *d*, Location of deletions³⁷. *e*, Positions of some genetic markers on the physical map.³⁷ A, J, *red α* , *red β* , *cI*, S and R are genes; *att* is the attachment region within which recombination takes place to integrate λ into the *E. coli* chromosome.

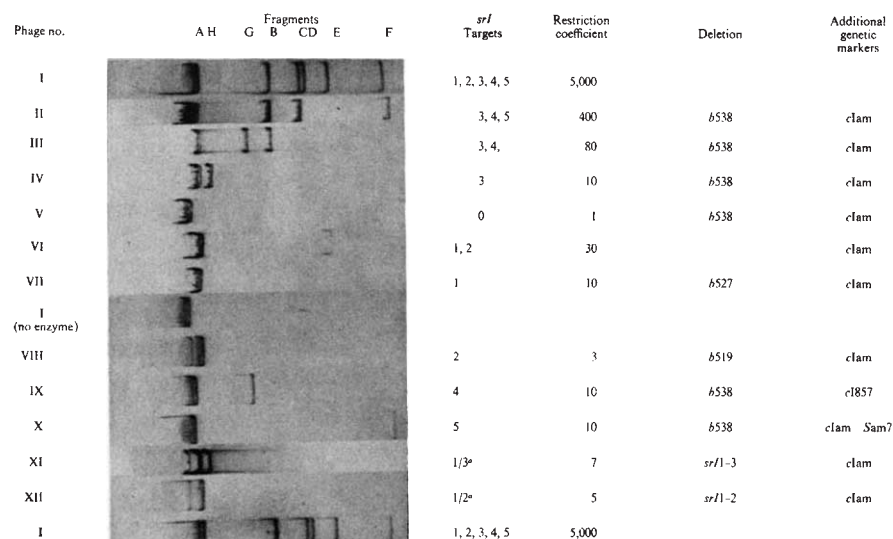


Fig. 2 Electrophoretic separation of *R.EcoRI* fragments of DNA from λ^+ and various derivatives. The location on the chromosome of targets for the enzyme, of the DNA fragments, and of deletions is shown in Fig. 1. The faint band visible behind the largest fragment results from adhesion of fragments by means of the natural cohesive ends of λ DNA. Superscript *a* denotes phage with hybrid targets generated by recombination *in vitro*. Although the two fragments of DNA from phages VII, VIII, and XII were not readily resolved by gel electrophoresis under the conditions used here, they have been clearly separated by equilibrium centrifugation in Cs_2SO_4 - HgCl_2 solutions. Phage were prepared from lysates (about 2l, titre usually 5.10^{10} p.f.u. ml^{-1}) made by infection of *E. coli* C600 in liquid culture. After two cycles of differential centrifugation (before and after treatment with RNase and DNase at $10 \mu\text{g ml}^{-1}$) the phage were resuspended in phage buffer (KH_2PO_4 , 0.3% w/v; Na_2HPO_4 , 0.7% w/v; NaCl , 0.5% w/v; MgSO_4 , 1 mM; CaCl_2 , 0.1 mM; gelatin 0.001% w/v), CsCl was added to 41.5% w/w and the solutions centrifuged to equilibrium. The phage band was removed with a Pasteur pipette, diluted with more CsCl in phage buffer, again centrifuged to equilibrium, and the band of phage collected after puncturing the bottom of the tube. Phage were stored in CsCl solution and DNA prepared as required by extraction of a diluted, dialysed solution with freshly distilled phenol (three extractions) followed by extensive dialysis against 0.01 M Tris-HCl (pH 8.0) 1 mM EDTA. DNA (0.3 to 1.0 μg depending upon the number and size of fragments generated) was incubated with *R.EcoRI*, prepared essentially as described by Yoshimori³⁸, in 20 μl . 0.01 M Tris-HCl (pH 7.5) 0.01 M 2-mercaptoethanol, 0.01 M MgCl_2 at 37°C for 30 min and the reaction stopped by addition of excess EDTA. The necessary quantity of enzyme (about 0.1 μl) was determined by titration against varying quantities of DNA and analysis by gel electrophoresis. The digests were heated in sealed capillaries at 70°C for 10 min, cooled rapidly at 0°C , mixed with 0.1% bromophenol blue in 50% w/v glycerol (2 μl) and applied to wells in 1% w/v agarose gels⁴⁰ (approx. $40 \text{ cm} \times 20 \text{ cm} \times 0.3 \text{ cm}$) containing $0.4 \mu\text{g ml}^{-1}$ ethidium bromide⁴¹ and after electrophoresis for about 16 h at a constant current of 40 mA the gels were photographed under ultraviolet light on Ilford FP4 film with a $4 \times$ red filter. For the *in vitro* recombination experiments, reaction mixtures of about 0.2 to 0.5 ml containing total DNA fragments, 10–30 $\mu\text{g ml}^{-1}$; Tris-HCl (pH 7.5) 66 mM; EDTA, 1 mM; MgCl_2 , 10 mM; NaCl , 40–80 mM; dithiothreitol, 10 mM; ATP, 0.1 mM; bovine serum albumin, 0.1 mg ml^{-1} ; T4 polynucleotide ligase (Miles Laboratories, Ltd), 0.5 u ml^{-1} ; were incubated at 10°C for 45 min and then kept on ice for 2–10 d before sampling for transfectants.

vention of Arber and Linn¹⁶, are within that part of the central, inessential region of the phage genome which is deleted in strain *b538*¹⁷ (see Fig. 1). Should the magnitude of restriction *in vivo*, as defined by the restriction ratio (that is the titre of non-modified phage assayed on the propagating host, divided by the titre on the restricting host), decrease as the number of restriction targets decreases^{18,19} then the deletion strain *b538* should be restricted less efficiently than λ wild-type. Furthermore, phages with even fewer restriction targets should have a selective advantage on transfer from a non-modifying host to a restricting host.

Loss of the two restriction targets in the deletion strain, *b538*, is indeed accompanied by a fall in the restriction ratio from 5,000 to approximately 400, concomitant with which is the predicted change in the pattern of DNA fragments observed on restriction of the DNA *in vitro* (Fig. 2). Fragments A, E and D of the wild-type strain (phage I) were replaced by a single large fragment, while fragments B, C and F were retained in the *b538* deletion strain (phage II). We attempted, therefore, to select for mutations resulting in the loss of the remaining restriction targets. Lysates of the deletion phage (phage II in Fig. 2) were made alternately on *E. coli* strain K and on a K strain harbouring the R1 plasmid²⁰. After seven such double cycles the restriction ratio had dropped from around 400 to 80 and after a total of eleven cycles the phage lysate comprised mainly restriction-resistant phages. Phages with restriction ratios of 80 (phage III) and 10 (phage IV) were isolated during the course of this enrichment and purified. Gel electrophoretic analyses of *R.EcoRI* digests of their DNAs (Fig. 2) showed that phage III differed from phage II in that fragments C and F had been

replaced by a larger fragment (G): we conclude that this phage had lost the right-most restriction target (*srI5*). DNA from the phage with a restriction ratio of 10 (phage IV) gave two large fragments: since fragments G and B were replaced by fragment H (Fig. 2) this phage must have retained only *srI3* rather than *srI4*. The DNA of the restriction-resistant phage (phage V) migrates as a single band in a position indistinguishable from that of whole λ DNA molecules. From a cross (Table 1, cross 1) of the restriction-resistant phage (phage V), to a suitably marked strain of phage λ having a full complement of restriction targets, phages having only the two left-most restriction targets were isolated. These phages have a low restriction ratio (~ 30), and the DNA fragments resulting from *in vitro* restriction (phage VI, Figs 2 and 3ii) included fragment E, as expected if phage VI had acquired *srI1* and *srI2*. Attempts to isolate spontaneous mutants lacking either *srI1* or *srI2* were unsuccessful because the selection favours deletions that remove both restriction targets. Phages having only *srI1* (phage VII) or only *srI2* (phage VIII) were therefore isolated as recombinants from crosses of phage VI (see Table 1) to the appropriately marked deletion strains *b527* and *b519*. The agarose gel does not readily separate the two large fragments expected when the DNA of either phage VII or phage VIII is restricted (Fig. 2) but these fragments were readily separated on caesium sulphate-mercuric chloride density gradients (data not shown). Phages having only *srI4* (phage IX) or only *srI5* (phage X) were isolated from a cross (Table 1, cross 4) by virtue of the close linkage of *srI4* to *cl* and of *srI5* to gene *S*. The restricted DNA from phage IX shows two fragments including that (G) expected as the sum of fragments F and C, while the restricted DNA from phage X again

shows two fragments, the smaller of which is fragment F (Fig. 2).

Separation and joining of DNA fragments

Fragments of DNA can be separated by gel electrophoresis but this method is inadequate for the preparative resolution of the larger fragments (Fig. 2). Instead, we turned to equilibrium centrifugation in caesium sulphate solutions containing mercuric chloride²¹ for this gave good separation of sheared fragments of λ DNA, enabling them to be placed in order on the λ chromosome²². These results (Fig. 1) show that the three fragments arising from breakage of phage VI DNA with

Table 1 Selection of recombinant phages

No.	Phage cross Genotypes of phages*						
	b538	3°	cIam	4°	+	5°	(Phage V)
1	1+ 2+	3+ cIts	4+ Pam	5+			
	1+ 2+	3° + cIam	4° 5°	+			(Phage VI)
2	1+ b527 3+ red cIts	3+ 5+	Ram				
	1+ 2+ 3° + cIam	4° 5°	+				(Phage VI)
3	b519 2+ 3+ red cIts	4+ 5+	Ram				
	h γ b538 3° cIam	4° 5°	+				(Phage V)
4a	h 80 3+ cIts	4+ 5+	Sam				
	h λ b538 3° cIam	4° 5°	+				(Phage V)
4b	h 80 3+ cIts	4+ 5+	Sam				

No. 1: *srI1*⁺*srI2*⁺ (that is *b538*⁺) *cIam* *P*⁺ recombinants were selected as phages of normal buoyant density giving clear plaques at 32° C on a suppressor-free (*sup*^o) host; those with *R*₁~30 were shown to have only targets *srI1* and *srI2* (Phage VI, Fig. 2). No. 2: *b527 red*⁺ *cIam* *R*⁺ recombinants were selected on a *sup*^o *polA* host²³ on a medium supplemented with sodium pyrophosphate (3 mM)¹⁷; most recombinants have *R*~10 and have only *srI1* (Phage VII, Fig. 2). No. 3: *b519 red*⁺ *cIam* *R*⁺ recombinants were selected on a *sup*^o *polA* host²³ on a medium supplemented with sodium pyrophosphate (3 mM)¹⁷; most recombinants have *R*~4, and have only *srI2* (Phage VIII, Fig. 2). No. 4a: *h λ b538 cI857 S*⁺ recombinants were selected as clear plaques at 39° C on a host resistant to ϕ h80 and carrying *supII*, which suppresses *cIam* but not *Sam*^{7,22}. A recombinant having *R*~10 was shown to have only *srI4* (Phage IX, Fig. 2). No. 4b: *h λ b538 cIam* phages were isolated as turbid plaques at 42° C (high temperature selects against ϕ h80 host range) on a *supIII* host after enriching for those phages (*Sam*7) unable to lyse a *supIII*^o host. (The unlysed cells were concentrated and the phage released by chloroform.) An *Sam*7 recombinant having *R*~10 has only *srI5* (Phage X, Fig. 2).

*Abbreviations used: *cIam*=*cIam*509; *cIts*=*cI857*, *Pam*=*Pam*80; *Ram*=*Ram*5; *Sam*=*Sam*7; *red*=*red*3; 1⁺=*srI1*; 2⁺=*srI1* etc. and 1°, 2° and so on refer to mutations conferring resistance to restriction.

†*R*=Restriction ratio=Titre on K strain
Titre on K/R1 strain

The media, methods of making and assaying phage stocks and crosses were as described previously^{18,21}. The standard *rec*⁺ host for phage crosses was QR47²³.

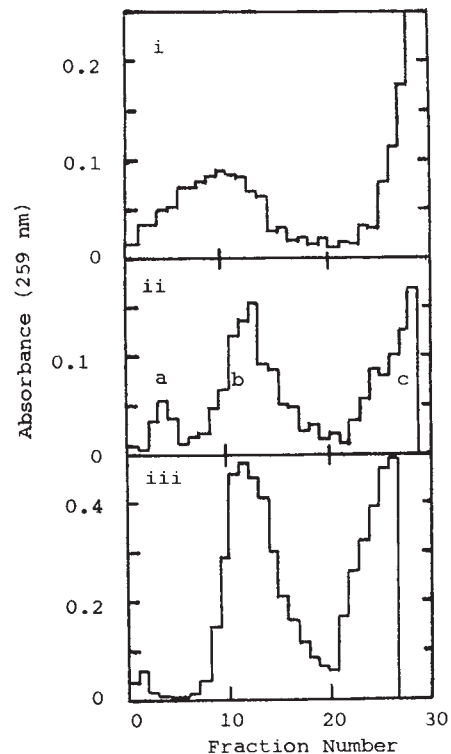


Fig. 3 Separation of DNA fragments in *R.EcoRI* digests by equilibrium centrifugation in Cs_2SO_4 - HgCl_2 gradients. Density increases from right to left. (i) Phage IV, (ii) Phage VI, (iii) Phage XII. Solutions of DNA (100 μg in 1.0 ml 0.01 M Tris-HCl (pH 7.5) 0.01 M 2-mercaptoethanol, 0.01 M MgCl_2) were digested with *R.EcoRI* (10 μl concentrated DEAE-cellulose fraction; 37° C, 1 h), extracted with an equal volume of freshly distilled phenol and dialysed twice against 0.3 M Na_2SO_4 in 5 mM sodium borate (pH 9.0) and then twice against 5 mM borate (pH 9.0). The solutions were then heated at 65° C for 10 min, cooled in ice, weighed and their DNA concentration was determined spectrophotometrically. Mercuric chloride solution (1 mM) was then added to give an *r*₀ of 0.22 (refs 21 and 22) and the solutions were diluted to 2.0 g with the borate buffer and Cs_2SO_4 added to 42.0% w/w. The solutions were transferred to 10 ml polypropylene centrifuge tubes, overlaid with liquid paraffin and centrifuged at 20° C at 30,000 r.p.m. for 60 h in an MSE SS65-II centrifuge, 8 $\times$ 40 Ti rotor with adaptors. Fractions of 4 drops were collected, diluted with 0.2 ml H_2O and analysed spectrophotometrically.

R.EcoRI would have quite different densities, as would the corresponding two fragments of phage IV DNA. Fragments of DNA from these phages were separated as predicted (Fig. 3i and ii) and recovered quantitatively.

Our first experiments on the joining of *R.EcoRI* fragments of λ DNA involved the construction of phage chromosomes with specific deletions so that the phage head would be able to accommodate additional DNA. We therefore confined our attention to phages with the targets *srI1*, *srI2* and *srI3* because removal of the DNA between either *srI1* and *srI2* or between *srI1* and *srI3* would affect only the inessential region of the λ chromosome. Receptor chromosomes were constructed from (a) the left and right fragments of phage VI DNA, and (b) the left fragment of phage VI DNA and the right fragment of phage IV DNA. In (a) the mixture of fragments in an *R.EcoRI* digest of phage VI DNA was used but in (b) the isolated fragments of each DNA were used. The mixtures of fragments were stored in the cold for 2 d under conditions favouring the annealing of the cohesive ends of *R.EcoRI* breaks¹² and incubated with phage T₄ polynucleotide ligase²³. After storage at 0° C for 2–10 d, samples were taken from the reaction mixtures for transfection of *E. coli* (ref. 24 and A. E. Jacob and S. J. Hobbs, personal communication) and the phages from the resulting plaques were isolated and investigated.

In a second series of experiments *R.EcoRI* fragments of DNA were inserted into the genomes of our receptor phages and

into that of phage IV, which possesses only *sr13* and carries the *b538* deletion. The central fragment of phage VI DNA, isolated from a caesium sulphate gradient (Fig. 3ii), and a DNA fragment carrying part of the *trp* operon of *E. coli*, isolated by agarose gel electrophoresis of an *R.EcoRI* digest of DNA (with a ^{32}P marker) from a λ *trp* phage (λ *trp13*, which had incorporated the bacterial *trpD* and *E* genes without deleting *sr13*)²⁰, were used.

Annealing of DNA fragments with cohesive ends would be expected to yield a range of products, each convertible into single DNA molecules, linear or circular, by polynucleotide ligase. SV40 DNA, for example, contains one target for *R.EcoRI* and gave monomeric and concatenated linear and circular molecules in these reactions¹². With λ DNA the situation is more complex because reactions will occur between the natural cohesive ends of the molecule²⁵ as well as the cohesive ends generated by *R.EcoRI*. The length and composition of the natural cohesive ends²⁶ relative to those created by *R.EcoRI*⁹ is such that the former would cohere preferentially. The reaction products used for transfection will, therefore, comprise a complex population of DNA molecules, though many of these would not yield viable phage, either because they are too large to be packaged or because they lack essential parts of the λ genome²⁷. In the experiments described here the yield of transfectants from the products of ligase reactions varied from 20 to 120 phage per μg DNA in the transfection mixture, compared with about 10^3 to 10^4 phage per μg from the corresponding DNA before restriction. (In subsequent experiments yields of transfectants from ligase reactions have been increased to about 5,000 per μg DNA and about 5×10^4 per μg from unrestricted DNA.) Omission of ligase reduced yields of transfectants from restricted DNA to about 1%, some of which appeared to result from joining of fragments *in vivo*.

Characteristics of the *in vitro* recombinants

(a) Receptor genomes: The three fragments from phage VI DNA could be joined to reconstitute parental phages or the two large fragments could be joined to give a phage lacking the DNA normally present between the two restriction targets (that is, ∇ *sr11-2*). Only one of twenty progeny made from the fragments of phage VI had the full complement of DNA, and could have resulted from the joining of all three fragments. The remaining 19 isolates showed an increased resistance to chelating agents¹⁷, as expected for phages with deletions. Eighteen of these nineteen phages had a restriction ratio characteristic of phage with a single restriction target. The remaining phage was restriction-resistant and could have resulted from joining of

fragments by means other than the short cohesive ends generated by *R.EcoRI*. The deletion within this phage must extend through *sr11* and *sr12* and yet this and the other phages were able to integrate into the *E. coli* chromosome to give stable lysogens as shown by their ability to form stable, immune colonies²⁸. (The alternative explanation that we have selected a deletion phage present in the original phage preparation seemed unlikely since the lysate had been purified by two cycles of equilibrium centrifugation in CsCl solution; furthermore most deletion mutants of phage λ are integration defective¹⁷.) The conclusion that the essential features of the attachment region are right of *sr12*, is strengthened by the fact that the new deletion phages (∇ *sr11-2*) showed normal site-specific recombination in phage crosses (Table 2). Gel analyses of the restricted DNA of one of these ∇ *sr11-2* phages (phage XII) confirmed that fragment E was missing: the two large fragments present were separable in a caesium sulphate-mercuric chloride gradient (Fig. 3iii). The buoyant density of phage XII indicates a deletion equivalent to 9.7% of the λ chromosome.

Phage formed by joining the left-hand fragment of phage VI and the right-hand fragment of phage IV lack the DNA normally present between targets *sr11* and *sr13* (that is ∇ *sr11-3*). The five phages isolated from such an experiment all had a low restriction ratio (~ 7), showed an increased resistance to chelating agents, failed to integrate into the *E. coli* chromosome and failed to form plaques on a *polA* host. The latter property²⁹ is characteristic of all recombination-deficient (*red*⁻) strains of phage λ and is consistent with the deletion of at least part of the *red* genes. Crosses of the new deletion phages to appropriately marked *red*⁻ strains of phage λ (Table 3, line 4) confirm that ∇ *sr11-3* phages are defective in the *red α* gene but not in the *red β* gene (see Fig. 1). These data provide genetic evidence that *sr13* is either within the *red α* gene or between *red α* and *red β* . Gel electrophoreses of the restricted DNA of one of the ∇ *sr11-3* phages (phage XI) confirm the presence of the two predicted DNA fragments and the buoyant density of phage XI indicates a deletion of 20.7% of the λ chromosome.

(b) Phages made from three fragments. The purified fragment E from phage VI was annealed with the restricted DNA from phage IV, which has only target *sr13*, and the mixture treated with ligase. Since phage IV has the *b538* deletion (see Fig. 1), it lacks the DNA of fragment E and, if *sr13* is within the *red α* gene, the insertion of DNA at this site will produce a *red*⁻ phage. We isolated 78 transfectants from the reaction mixture and detected six defined as *red*⁻ by their failure to form plaques on a *polA* host²⁹. All six *red*⁻ phages were characterised as

Table 2 Site specific recombination in phage crosses

Cross*					Frequency of site specific recombination (Twice the frequency of turbid (<i>c</i> ⁺) <i>J</i> ⁺ <i>R</i> ⁺ recombinants)
<i>Jam6</i>	<i>att</i> ⁺	<i>red3</i>	<i>c</i> ⁺	+	
-----					1.33%
+	<i>att</i> ⁺	<i>red3</i>	<i>c1857</i>	<i>Ram5</i>	
<i>Jam6</i>	<i>att</i> ⁺	<i>red3</i>	<i>c</i> ⁺	+	0.024%

+	<i>b538 (att ∇)</i>	<i>red3</i>	<i>c1857</i>	<i>Ram5</i>	1.46%, 1.37%, 1.44%**
<i>Jam6</i>	<i>att</i> ⁺	<i>red3</i>	<i>c</i> ⁺	+	

+	<i>sr11-2</i>	<i>red3</i>	<i>c1857</i>	<i>Ram5</i>	

+	<i>sr11-2</i>	<i>red3</i>	<i>c1857</i>	<i>Ram5</i>	

* These crosses of *red*⁻ phages were made in a *recA* host (QR48)³³.

** Three different ∇ *sr11-2* recombinants were used, one of which was restriction resistant and another was a recombinant made from purified fragments.

Table 3 The *red* functions of *in vitro* recombinants

Test phage	Recombination frequency (%) between immunity and gene <i>R</i> expressed as twice the frequency of <i>i</i> ⁴ <i>R</i> ⁺ recombinants	
	(a) Cross to <i>redα i</i> ⁴ <i>Ram5</i>	(b) Cross to <i>redβ i</i> ⁴ <i>Ram5</i>
λ ∇ <i>srI1-2 i</i> ⁴ <i>R</i> ⁺ (<i>red</i> ⁺)	7.2	2.1
λ <i>bio69 i</i> ⁴ <i>R</i> ⁺ (<i>redα</i> ⁻)	0.02	1.6
λ <i>red3 i</i> ⁴ <i>R</i> ⁺ (<i>redβ</i> ⁻ polar on <i>redα</i>)	1.4	0.02
λ ∇ <i>srI1-3 i</i> ⁴ <i>R</i> ⁺ †	0.02; 0.02	2.3; 2.4
λ <i>b538 srI3 red</i> ⁻ <i>R</i> ⁺ ‡	0.02; 0.01; 0.01; 0.02; 0.02; 0.02.	2.8; 3.0; 2.3; 2.8; 2.9; 2.1.

* The abbreviation *i*⁴ is used for the immunity of phage 434.

† 2 different *srI1-3* deletion phages.

‡ The six *red* phages resulting from insertion of DNA at *srI3*.

The *Red* phenotypes of the phages were deduced from the frequencies of *i*⁴*R*⁺ recombinants generated in crosses to a *redα* phage (λ *bio69 i*⁴*Ram5*) and to a *redβ* phage (λ *redβ113 i*⁴*Ram5*)³⁹. All crosses were made in the *recA* host, QR48³³, and the recombinants selected on a *sup*⁰ (λ *i*⁴*Ram5*) strain of *E. coli*.

redα⁻*redβ*⁺ (Table 3, line 5). Five of these six resembled the original phage IV in having a restriction ratio of around 10, as expected for a phage with only one restriction target: the remaining *red*⁻ phage (Phage XIII) had a restriction ratio of 80, suggesting two restriction targets. This phage meets the predictions for a recombinant in which the whole of fragment E had been inserted at *srI3*. Gel electrophoresis of the restriction products of the DNA from phage XIII (Fig. 4) shows two large DNA fragments like those of phage IV and a third fragment of the same size as fragment E.

One of the other five phages was investigated further. This phage (Phage XIV) had a buoyant density less than that of the receptor phage (Phage IV) and analysis by gel electrophoresis of the restricted DNA from this phage showed one fragment moving in the same position as the smaller fragment of the receptor DNA and a slower fragment moving slightly faster than the larger fragment of the receptor genome. The formation of this and the other *red*⁻ phages with only one restriction target can be explained if fragment E joined to one end of the receptor chromosome via its cohesive end and then at the other end via an illegitimate recombination event³⁰. In the case of phage XIV the illegitimate recombination event was associated with the deletion of more than 10% of the phage DNA. This explanation for the origin of those *red*⁻ phages having a single restriction target is supported by a control experiment in which fragment E was not added to the receptor DNA and here the ten *red*⁻ phages isolated were all restriction resistant.

A fragment of DNA containing the *trpD* and *E* genes of the *trp* operon of *E. coli* together with their operator and promoter was isolated and purified from an *h*⁸⁰*i*⁴*cI* *trp*-transducing phage (λ *trpI3*)²⁰. This fragment was fused to the restriction products of the receptor phage ∇ *srI1-2* (Phage XII) and among 120 transfectants we detected³¹ one *h*⁴ *i*⁴ *cIam* phage having the

trpD and *E* genes. This λ *trp* phage expresses the *trp* genes from the *trp* promoter and from a λ promoter (N. E. M. and W. J. Brammar, unpublished observation). Like four of the five recombinant *red*⁻ phages described above, this phage has a low restriction ratio (*R* ~ 10) and has not incorporated the whole of the DNA fragment. In fact, the phage has become integration defective consistent with loss of DNA to the right of *srI2*. The buoyant density of the new λ *trp* phage indicated that it has about 3% more DNA than λ wild type. Had the whole fragment been incorporated into the receptor genome then a phage with at least 10% more DNA than λ wild type would be expected. At least two other phages had acquired extra DNA but did not contain the *trp* genes.

Practical implications

Our results show that the phage λ genome can be used as a receptor for genetic material that cannot be incorporated by conventional genetic techniques. The biochemical approach used provides a convenient means of cloning DNA, but more importantly it lends the extensive genetic and biochemical experience acquired with phage λ³⁴ to the study of the additional genetic material. This DNA can be inserted into the phage chromosome so that its expression is controlled autonomously, or by the phage regulatory systems⁴², when advantage can be taken of the efficient λ promoter, P_L, and *N*-gene protein which allows RNA polymerase to read through transcriptional stops⁴³. By appropriate choice of the phage genotype the yields of gene products can be greatly enhanced³⁵; experiments with λ *trp* phages, for example, have shown that the five enzymes of the *trp* operon can be boosted to comprise as much as 50% of the soluble protein of the host cell (A. Moir and W. J. Brammar, personal communication). The insertion of appropriate DNA fragments into marked receptor phages offers new approaches for extending the biochemical genetics of eukaryotic systems. We have shown that eukaryotic DNA can be inserted into the λ receptor phages (unpublished results). It has been established that eukaryotic DNA can be transcribed within the *E. coli* cell³⁶, but it is not known whether a eukaryotic messenger RNA is translated.

We expected that transfectant phages would result from covalently linked fragments of DNA as either linear or circular chromosomes³⁴ because a linear molecule could circularise by joining of free cohesive ends after its entry into the bacterial cell. Our results suggest that the bacterial cell may provide an alternative way of joining two non-homologous regions of DNA and in so doing generate deletions. Further experiments have proved that deletion phages are indeed created rather than merely selected (N. E. M. and K. M., unpublished observations) and they confirm the use of this method for the production of localised deletions. Illegitimate recombination events were first suspected because restriction targets were lost; those deletions generated in the region of *srI2* have been characterised further as integration proficient, integration defective, or integration and recombination defective (K. Borck and N. E. M., unpublished observation). Lai and Nathans⁴⁴ have detected an analo-

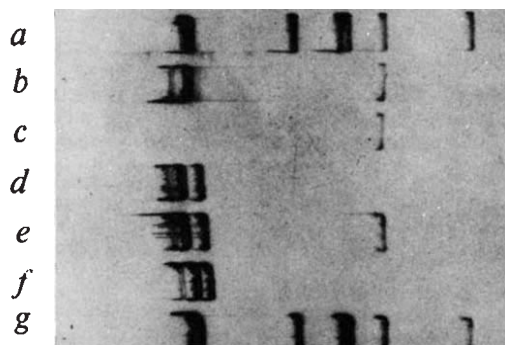


Fig. 4 Agarose gel electrophoresis of R.*EcoRI* digests of DNA used in and obtained from recombination experiments *in vitro*. Experimental details were the same as described in the legend to Fig. 2. (a) Phage I; (b) Phage VI; (c) Fragment E purified from phage VI (peak a from Fig. 3ii); (d) Phage IV; (e) Phage XIII, (f) Phage XIV (Phages XIII and XIV are the recombinant phages); (g) Phage I. The faint, slowest-moving band visible in (b), (d), (e) and (f) results from adhesion of the large fragments of DNA via their normal cohesive ends.

gous class of deletion mutants following the *in vivo* association of restriction fragments of SV40 DNA and have made elegant use of these deletions to further the genetic analysis of the SV40 virus.

Insertion of DNA fragments into a receptor chromosome via illegitimate recombination destroys the RI restriction target, and can therefore be a disadvantage if one is concerned with the recovery of a cloned DNA fragment. It does provide, however, a means of incorporating part of the DNA from a fragment that is too large to be accommodated within the phage genome. Removal of the DNA between *srI1* and *srI3* in the presence of a deletion between genes *P* and *Q* (*ninR5*)⁴⁵ eliminates 27% of the λ DNA. Since phage λ can accommodate about 10% more than its normal complement of DNA these deletions permit the insertion of DNA equivalent to about 15 genes. Although we have not used them in these experiments, powerful methods are available for selecting only those phages that have gained DNA.

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Glutamine synthetase and ammonium regulation of nitrogenase synthesis in *Klebsiella*

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Glutamine synthetase seems to act as a positive control element for nitrogenase synthesis in a way similar to that already proposed for histidase synthesis in Klebsiella. Studies along lines opened up by these findings may provide answers to many of the problems in establishing nitrogen fixation in non-leguminous plants.

In both fungi and bacteria, synthesis of enzymes involved in the utilisation of nitrogen sources such as amino acids, purines, nitrate and nitrite, is normally repressed when sufficient ammonium nitrogen for growth is provided in the culture medium. Arst and Cove¹ suggested that ammonium repression of all such enzymes in *Aspergillus nidulans* was effected by a single mechanism: the *areA* gene product, as yet unidentified biochemically, seems to be essential for enzyme synthesis but is absent, or cannot function, in the presence of NH_4^+ . Prival, Brenchley and Magasanik^{2,3} have

studied utilisation of histidine and regulation of histidase and other enzymes of the *hut* operons in *Klebsiella aerogenes*. Histidine can serve as nitrogen source or carbon source; histidase is subject to both ammonium repression and glucose (carbon-catabolite) repression and the relief of either derepresses histidase synthesis (see ref. 2). Derepression of histidase synthesis under limiting nitrogen is independent of cyclic AMP but requires high levels of glutamine synthetase; mutants lacking glutamine synthetase fail to derepress synthesis of histidase under these conditions³. Moreover, in mutants which are constitutive for glutamine synthetase, histidase and proline oxidase (the first enzyme of proline catabolism) escape from ammonium repression³. Purified unadenylylated glutamine synthetase, but not the fully adenylylated enzyme, activates correct transcription of *hut* DNA *in vitro*; glutamine synthetase is as effective in this respect as cyclic AMP plus catabolite activating protein (CAP) and has been proposed therefore as a positive control

Table 1 Growth of *K. pneumoniae* M5a1 and mutant RT4 on various compounds as sole N source

	Nitrogen-source	M5a1	RT4
a	(NH ₄) ₂ SO ₄	+++++	++++
	Glutamine	+++++	++++
	Asparagine	+++++	+
	Glutamate	++	—
	Aspartate	++++	++
	Alanine	++++	++++
	Arginine	++	—
	Histidine	++++	—
	Proline	++	—
	Nitrate, sodium	+++++	—
	Nitrite, sodium	+++++	—
	Urea	+++++	++++
	Adenine	+++++	—
	Hypoxanthine	++++	—
	Xanthine	++	—
b	N ₂	+++	—
c	Casein hydrolysate	+++	+++

Nutrient broth cultures of M5a1 and RT4 were centrifuged, washed, resuspended in saline-phosphate and plated out on minimal medium containing, g l⁻¹, 20 glucose, 3.40 KH₂PO₄, 12.06 K₂HPO₄, 0.025 Na₂MoO₄·2H₂O, 0.025 FeSO₄, 0.1 MgSO₄, supplemented where indicated with 1 g of nitrogen source. *a*, Growth was estimated visually after 48 h at 30° C in air on a scale ranging from — (no growth) to + + + + + (very good growth). *b*, Growth was estimated similarly after 5 d incubation at 30° C in an anaerobic bag¹² with a throughput of N₂/CO₂ (99:1). No nitrogen source was included in the medium. *c*, Growth estimated from overnight liquid cultures grown under argon.

element for transcription of *hut* DNA⁴.

Nitrogenase synthesis in *Klebsiella pneumoniae* is repressed by NH₄⁺ (ref. 5); there is no evidence for glucose repression and this is reasonable since N₂, unlike histidine, can serve only as a nitrogen source. Here I report: (i) the characterisation of a mutant of *K. pneumoniae* which was altered in its regulation of glutamine synthetase, had lost the ability to grow on a large number of compounds as sole nitrogen source and failed to synthesise both nitrogenase components; (ii) the transfer of nitrogen fixation (*nif*) genes to glutamine synthetase-constitutive mutants of *K. aerogenes* resulting in constitutive expression of *nif*. These results strongly implicate glutamine synthetase as being involved in ammonium regulation of nitrogenase synthesis.

Pleiotropic mutation which blocks nitrogenase synthesis

Mutant strain RT4 was obtained by mutagenesis of *K. pneumoniae* M5a1 with IPMS (isopropylmethane sulphonate)⁶ followed by selection with ampicillin and D-cycloserine for glutamine auxotrophs. RT4 grew well on glutamine-supplemented minimal medium; grew well, but with a longer

lag, on unsupplemented minimal medium (with (NH₄)₂SO₄ as sole nitrogen source) and failed to grow on NH₄⁺-free medium (NFM) with histidine, nitrate or N₂ as sole nitrogen source. The mutation was, in fact, extremely pleiotropic resulting in loss of the ability to utilise many nitrogen sources (Table 1) and was superficially very similar to the *asm*⁻ mutation described by Nagatani, Shimizu and Valentine⁷; such *asm*⁻ mutants produce low levels of glutamate synthase^{7,10} (glutamine-amide-2-oxoglutarate amino transferase). This enzyme is responsible for the second step in the glutamine synthetase-glutamate synthase pathway, the primary route of NH₄⁺-assimilation in *Klebsiella* grown with low NH₄⁺ or poor nitrogen sources⁷⁻¹⁰. Although *asm*⁻ mutants fail to grow on N₂ as sole nitrogen source, Streicher *et al.*¹¹ mentioned that nitrogenase, as determined by the acetylene reduction test, is induced; nitrogenase levels are comparable to the wild-type level in aspartate-supplemented medium. RT4, however, showed no acetylene reduction in Pankhurst tube tests^{13,14} on NFM with either vitamin-free casein hydrolysate (160 µg ml⁻¹) or L-glutamine (100 µg ml⁻¹) as nitrogen source. It was possible that NH₄⁺ produced from these nitrogen sources was not readily assimilated and therefore accumulated to repress nitrogenase synthesis. In neither case, however, was NH₄⁺ detectable in the culture supernatant at the end of the test.

Comparison of nitrogenase, glutamine synthetase, glutamate synthase and glutamate dehydrogenase activities in RT4 and M5a1 confirmed that RT4 was not *asm*⁻ (Table 2). Cultures were grown in conditions which allowed derepression of nitrogenase synthesis yet in which growth was independent of nitrogen fixation and in which Nif⁺ revertants of Nif⁻ RT4 had no selective advantage. Glutamate synthase activity in RT4 was very similar to that in M5a1. But, glutamine synthetase activity (—Mg²⁺) was 40% lower and below the 0.7 units of activity required for the escape of histidase synthesis from ammonium repression³. Glutamate dehydrogenase was 50% higher. Nitrogenase activity was again absent from whole organisms of RT4. When ATP + dithionite-dependent nitrogenase activities were assayed in anaerobically prepared cell-free extracts of RT4, both component activities were absent (Table 3). Brenchley, Prival and Magasanik¹⁰ showed that levels of glutamine synthetase and glutamate dehydrogenase varied inversely in *Klebsiella aerogenes*. During growth on low NH₄⁺, glutamine synthetase level is high, glutamate dehydrogenase low; ammonium assimilation through glutamine synthetase and glutamate synthase is favoured. When NH₄⁺ is in excess, glutamine synthetase is low, glutamate dehydrogenase high: ammonium assimilation can proceed through glutamate dehydrogenase. Brenchley *et al.*¹⁰ suggested that glutamine

Table 2 Nitrogenase, glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) activities of M5a1, mutant RT4 and revertants of RT4

Strain	Relevant phenotype	Nitrogenase*	—Mg ²⁺	GS† +Mg ²⁺	% +Mg ²⁺ /—Mg ²⁺	GOGAT‡	GDH‡
M5a1	Hut ⁺ Nif ⁺	38	0.99	1.34	135	92	79
RT4	Hut ⁻ Nif ⁻	0	0.60	1.02	170	97	125
RT48	Hut ⁺ Nif ⁺	24	0.73	1.13	155	—	—
RT49	Hut ⁺ Nif ⁻	0	0.64	1.06	166	—	—

Cultures were grown at 28° C on NFM supplemented with 1.2 mg casein hydrolysate ml⁻¹ and sparged with argon. When a culture reached approximately 0.25 mg bacterial protein ml⁻¹ (40 EEL Spectra units at 540 nm) a sample was removed and assayed for whole-cell acetylene reduction⁵. Cell-free extract was prepared from the rest of the culture as described by Prival *et al.*³ except sonication was with a Dawe Soniprobe for 4 × 15 s at 2.5 A with 1 min cooling intervals. Glutamine synthetase activities were measured by 'transferase' assays¹⁵ with and without 60 mM MgCl₂ (ref. 3). Assaying in the absence of Mg²⁺ gives a measurement of total glutamine synthetase (both adenylylated and unadenylylated subunits are active) whereas assaying in the presence of Mg²⁺ gives a measurement of unadenylylated enzyme which is believed to be the biosynthetically active form *in vivo*. MgCl₂, although it inhibits adenylylated subunits, appears to stimulate activity of unadenylylated glutamine synthetase in crude extracts (see ref. 3). Activities measured with Mg²⁺ therefore overestimate *in vivo* glutamine synthetase activity: '% +Mg²⁺/—Mg²⁺' overestimates % unadenylylated enzyme. Glutamate synthase and glutamate dehydrogenase activities were measured at 20° C by following NADPH oxidation at 340 nm (ref. 9) with a Unicam SP1800 spectrophotometer. Glutamate synthase activities here have been corrected by subtracting 10% of the measured glutamate dehydrogenase activity¹⁰.

*nmol C₂H₄ reduced per min per mg bacterial protein.

†µmol γ glutamylhydroxamate formed per min per mg protein.

‡nmol NADPH oxidised per min per mg protein.

— = not determined.

synthetase repressed synthesis of glutamate dehydrogenase; thus the higher level of glutamate dehydrogenase in RT4 (Table 2) may be a consequence of an inability to derepress full synthesis of glutamine synthetase.

Glutamine synthetase levels in RT4 and M5a1 were compared in extracts from cultures grown under both depressive conditions (glutamine as sole nitrogen source) and repressive conditions (glutamine and NH_4^+ as nitrogen sources). RT4 constitutively synthesised glutamine synthetase as a level intermediate between repressed and derepressed M5a1 (Table 4). It follows that the mutation in RT4 must directly affect regulation of glutamine synthetase synthesis; a mutation causing increased intracellular NH_4^+ production by some other pathway would explain a low level of glutamine synthetase when derepressed, but not the high level when repressed. Glutamine synthetase activities in M5a1 were comparable to those reported by Prival *et al.*³ in *K. aerogenes* MK-53. In agreement with these authors, the adenylation of glutamine synthetase was increased to compensate for overproduction (see RT4 grown with NH_4^+ and L-glutamine, Table 4). Moreover, glutamine synthetase was more unadenylylated in RT4 grown on L-glutamine where overall levels of enzyme were lower than in M5a1.

Table 3 Nitrogenase activities of M5a1 and RT4

Strain	Whole cell	Extract	I*	II†
M5a1	31	28	37	30
RT4	0.0	0.0‡	0.0	0.0

Activities are expressed as nmol C_2H_2 reduced per min per mg protein. Cultures were grown and whole-cell activities measured as described under Table 2. Cell-free extracts were prepared anaerobically from spheroplasts by combined osmotic shock and Triton-X-100 lysis (Christina Kennedy, personal communication). ATP+ dithionite-dependent nitrogenase activity was assayed in extracts as described by Eady *et al.*¹⁶ 0.5–1.0 mg of protein was used per assay.

*Component I (Fe-Mo protein) activity: 50 μg purified component II added to assay.

†Component II (Fe protein) activity: 50 μg purified component I added to assay.

‡When RT4 extract was assayed in the presence of both components I and II, no inhibition of the activity of the added purified nitrogenase components was observed: lack of nitrogenase activity in RT4 was not a consequence of enzyme inhibition.

Spontaneous 'revertants' of RT4 were obtained at a frequency of about 1 in 10^8 organisms by plating on NFM or NFM supplemented with 1 mg L-histidine or KNO_3 per ml. Clones obtained on NFM-histidine were either prototrophic or revertants which grew on histidine as sole nitrogen source but not on nitrate or N_2 . Similarly, clones obtained on NFM-nitrate were either prototrophic or revertants which grew on nitrate but not on histidine or N_2 . But, all of 62 clones obtained on N_2 were prototrophic; repeated attempts failed to produce a Nif^+ revertant which did not grow on histidine or nitrate. Glutamine synthetase activities of typical RT4 derivatives are shown in Tables 2 and 4; RT48 was a typical prototrophic Hut^+ revertant with wild-type glutamine synthetase activities and regulation; RT49 had regained only the ability to grow on histidine as sole nitrogen source and showed glutamine synthetase activities like RT4. Apparently derepression of *hut* enzymes had become independent of high levels of glutamine synthetase in this strain.

Glutamine auxotrophs of *K. aerogenes* have lesions which map at two separate loci, *glnA* and *glnB* (ref. 3). Most revertants of a *glnB* strain produce glutamine synthetase constitutively; these revertants (GlnC^- strains) have a second mutation at a site cotransducible with *glnA* (ref. 3). An F' *nif-his* plasmid (FN68) which also confers carbenicillin-resistance¹⁷ was transferred to RT4 from *Escherichia coli* JC5466 (provided by Dr R. A. Dixon). Nitrogenase activity was not restored to RT4 by this means so, unlike most *nif* mutations¹¹ the mutation in RT4 did not appear to be linked to *his*. The map position of the RT4 mutation is currently

Table 4 Regulation of glutamine synthetase in M5a1, RT4 and derivatives

Strain	Composition of medium		Glutamine synthetase		
	NH_4^+	L-Gln	− Mg^{2+}	+ Mg^{2+}	% + Mg^{2+} /− Mg^{2+}
M5a1	—	+	1.42	0.43	30
M5a1	+	+	0.24	0.20	83
RT4	—	+	0.62	1.04	168
RT4	+	+	0.61	0.27	44
RT48	—	+	1.17	0.42	36
RT48	+	+	0.27	0.22	81
RT49	—	+	0.63	0.96	152
RT49	+	+	0.60	0.25	42

(NH_4)₂SO₄ and L-glutamine were used at 1 g^{−1}; glucose at 5 g^{−1}. Overnight cultures, grown up under air on an orbital shaker, were suitably diluted into fresh medium, grown for 2–3 doublings and harvested from log phase. Extracts were prepared and glutamine synthetase assayed as described under Table 2.

being sought; this work will be reported in a subsequent communication.

Constitutive synthesis of nitrogenase

Prival *et al.*³ showed that GlnC^- strains which are fully constitutive for glutamine synthetase produce depressed levels of histidase and proline oxidase in the presence of NH_4^+ ; similar strains derived from nitrogen-fixing *K. pneumoniae* M5a1 might be constitutive for nitrogenase synthesis. If the RT4 mutation was in the *glnB* site, GlnC^- strains might be found among revertants of RT4. Over 80 prototrophic clones obtained by reverting RT4 to a Hut^+ or Nif^+ phenotype were checked for the production of a yellow-brown pigment when grown on tryptophan plus NH_4^+ as nitrogen sources. Pigment production under these conditions is characteristic of GlnC^- *K. aerogenes*³. No GlnC^- revertant of RT4 was identified by this means: either the test was not applicable to *K. pneumoniae* M5a1 or mutation to GlnC^- does not repair RT4. Additionally, 25 out of 25 Nif^+ revertants of RT4 tested failed to reduce acetylene when anaerobically grown in the presence of 1 mg (NH_4)₂SO₄ per ml.

K. aerogenes strain MK-53 and its GlnC^- derivatives MK-94 and MK-142 (see ref. 3) do not naturally fix nitrogen. The F' *nif-his* factor (FN68) which carries the *nif* genes of *K. pneumoniae* M5a1 (ref. 17) was therefore introduced into these strains to determine the effect of constitutive synthesis of glutamine synthetase on expression of *nif*. Significantly, Nif^+ His^+ exconjugants obtained by introducing the F' *nif* into *his*[−] derivatives of the GlnC^- *K. aerogenes* strains MK-94 and MK-142 retained 10–20% of their derepressed level of nitrogenase activity (Table 5) when grown on NH_4^+ . This partially constitutive synthesis of nitrogenase was specifically associated with the GlnC^- character since it was not shown by *K. aerogenes* MK-53 (FN68) or *K. pneumoniae* M5a1. Mutants of *Azotobacter vinelandii* which show 25% of their derepressed level of nitrogenase in the presence of ammonium have been described¹⁸ but have not been characterised with respect to glutamine synthetase.

Glutamine synthetase and nitrogen fixation

The possibility that synthesis of both nitrogenase and glutamine synthesis is positively regulated by an unidentified factor which is defective in RT4 and unaffected by NH_4^+ in GlnC^- strains cannot be ruled out completely. But it is difficult to see how a common defective regulator in RT4 could produce both low constitutive synthesis of glutamine synthetase and no synthesis of nitrogenase. It seems most likely, therefore, that glutamine synthetase itself is required as a positive control element for nitrogenase synthesis in the same way as has been proposed for histidase synthesis in *Klebsiella*^{3,4}; a direct demonstration of activation of transcription of *nif* by glutamine synthetase *in vitro* is still required. That nitrogenase synthesis only partially

Table 5 Expression of *nif* in GlnC⁻ (glutamine synthetase-constitutive) hosts

Organism	Strain	Derivation		Relevant phenotype	Nitrogenase activity (nmol C ₂ H ₄ reduced min ⁻¹ mg ⁻¹)	
		Mating performed	Character selected		NH ₄ ⁺	Grown on Aspartate
<i>K. pneumoniae</i>	M5a1	—	—	GlnC ⁺	<0.01	} 39-55*
<i>K. aerogenes</i>	MK-53 (FN68)	MK-53	Carbenicillin resistance	GlnC ⁺	<0.01	
		×				
		JC5466 (FN68)				
<i>K. aerogenes</i>	MK-94 <i>his</i> (FN68)	MK-94 <i>his</i>	His ⁺	GlnC ⁻	6.0	
		×				}
		JC5466 (FN68)				
<i>K. aerogenes</i>	MK-142 <i>his</i> (FN68)	MK-142 <i>his</i>	His ⁺	GlnC ⁻	7.5	
		×				}
		JC5466 (FN68)				

K. aerogenes MK-53, MK-94 and MK-142 (=MK-140) are published strains³. MK-94 *his* and MK-142 *his* were derived by IPMS mutagenesis and subsequent antibiotic selection for histidine auxotrophs. For matings using *E. coli* JC5466 (FN68) as donor, 0.1 ml of overnight broth cultures of both donor and recipient were spread together on nutrient agar. After 6-7 h organisms were scraped off, resuspended in saline-phosphate buffer at 10⁸-10⁹ organisms ml⁻¹ and 0.1 ml plated out on selective medium. The Nif⁺ *K. aerogenes* hybrids and *K. pneumoniae* M5a1 were grown under a stationary argon gas phase on NFM supplemented with 1mg (NH₄)₂SO₄ or aspartate ml⁻¹. When a culture reached 0.15-0.20 mg bacterial protein ml⁻¹ a sample was removed and assayed for nitrogenase activity (see legend to Table 2).

*Dependent on age of culture.

escapes ammonium repression in GlnC⁻ strains may reflect a specific requirement for highly deadenylylated glutamine synthetase. GlnC⁻ strains contain derepressed levels of glutamine synthetase in the presence of excess NH₄⁺ but the enzyme is more adenylated than under nitrogen limiting conditions (see ref. 2). If the adenylating 'cascade' system for glutamine synthetase^{19,20} can be blocked by mutation, such a mutation in a GlnC⁻ strain may produce fully constitutive nitrogenase synthesis.

If glutamine synthetase does exert a direct effect on *nif* expression, proposed transgenesis²¹ of repressible *nif* genes from *Klebsiella* to plants would require homologous glutamine synthetase structural and regulatory genes to be transferred along with *nif*. This problem could be avoided if *nif*-constitutive strains in which nitrogenase synthesis was independent of activation by glutamine synthetase could be obtained. If *nif* genes are in a single operon, one might have expected to obtain this class of mutants among Nif⁺ revertants of RT4. That such attempts failed may mean that *nif* genes in *Klebsiella* are located in more than one operon.

Duncan and Tierney²² have recently succeeded in picking up *nif* genes from *Rhizobium trifolii* thereby adding conviction to the widespread, but unsubstantiated, belief that genetic material from rhizobia codes for nitrogenase proteins in the nitrogen-fixing legume symbiosis. No *Rhizobium* has been shown to fix nitrogen in the free-living state. Duncan and Tierney²², however, obtained expression of presumptive *Rhizobium nif* genes by R-factor-mediated conjugation into *K. aerogenes* strain 418, naturally a non-fixer. In addition, expression of *Rhizobium nif* in *K. aerogenes* was repressed by NH₄⁺. It is tempting to speculate that free-living rhizobia do not synthesise nitrogenase because *Rhizobium* glutamine synthetase is unable to activate transcription of *nif* DNA by *Rhizobium* RNA polymerase. *R. japonicum* glutamine synthetase is known to be immunologically distinct from the glutamine synthetases of the coliform bacteria, *E. coli*, *K. pneumoniae* and *Salmonella typhimurium*²³. Nitrogenase is synthesised, however, within the root nodules of leguminous plants; either the plant provides suitable 'machinery' for transcription of *nif* or changes occur in *Rhizobium* RNA polymerase during establishment of effective symbiosis, such that *nif* genes can be transcribed in the absence of activation by glutamine synthetase. Possible answers to questions raised by studies on symbiotic nitrogen fixation therefore can be suggested from basic knowledge of regulation of synthesis of nitrogenase in *K. pneumoniae*; such information must also

greatly increase the chances of success in any attempt in the future to establish nitrogen fixation in non-leguminous crop plants.

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letters to nature

Possible antineutrino pulse of extraterrestrial origin

WE report an unusual event observed during our search for electron antineutrino $\bar{\nu}_e$ bursts of extraterrestrial origin¹. Our experiment uses a deep underground $\bar{\nu}_e$ burst-detector station, adjacent to the Brookhaven Solar Neutrino Observatory² at a depth of 4,400 metres water equivalent (m.w.e.) in the Homestake Gold Mine in Lead, South Dakota. At this station we have placed a number of large water Cerenkov

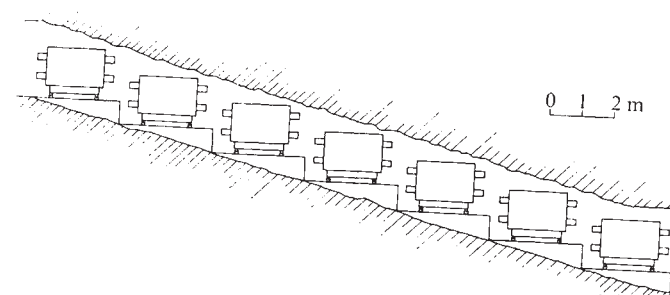


Fig. 1 Arrangement of water Cerenkov counters in a tunnel 1,480 m below surface in the Homestake Mine in Lead, South Dakota. The lowest right hand counter is No. 1 and the highest left hand one is No. 7.

counters (Fig. 1) designed to detect $\bar{\nu}_e$ interactions by the Cerenkov radiation from positrons resulting from $\bar{\nu}_e + p \rightarrow n + e^+$ (ref. 3).

Each Cerenkov counter is a 2 m³ cylinder, 1.78 m long by 1.22 m filled with deionised water containing Amino-G wave length shifter and viewed by eight photomultipliers 12.5 cm in diameter. The majority logic coincidence circuit associated with each counter gives an output pulse whenever five or more of the eight photomultipliers viewing that counter give coincident signals. The occurrence of either a single large amplitude pulse from any one counter or of two or more counter pulses within 0.1 s initiates a magnetic tape recording of the time distribution of the pulses in each counter, the summed photomultiplier pulse amplitude associated with each pulse and the

photomultiplier pattern of each counter involved in the coincidence. The time difference between pulses within an event is measured with a 20 MHz oscillator providing 50 ns time resolution for these differences.

On January 4, 1974, at 17 h 16 min 48 s GMT we observed an event involving a total 24 pulses within 3 ms in six of our detectors. These pulses occurred in four groups, each $\sim 1 \mu\text{s}$ wide. The first and second groups were separated by 640 μs , the second and third by 968 μs and the third and fourth by 928 μs . The first group consisted of nine pulses distributed over the six counters, the second group involved six pulses and the third group eight pulses. These three groups used up 23 of the 24 available readout channels leaving only one readout channel available for the last pulse. From our detector thresholds and pulse height data, we estimate the energy of the particles involved in these pulses to be $20 \text{ MeV} \leq E \leq 100 \text{ MeV}$. These pulse heights indicate that the charged particles responsible for these pulses had ranges considerably less than half of the diameter of our counters and so are unlikely to have traversed our counters.

Since our readout channels were completely filled by the four recorded bursts we do not know if there were any subsequent pulses. We only know that 0.8 s after the first pulse, when our transfer to tape was completed, there were no more counts, so that the pulse duration period was less than this time.

Figure 2 shows the time distribution of the observed bursts and Fig. 3 gives the pulse distribution within each burst. Details of the burst data are given in Table 1.

We have considered and eliminated the possibility that this event is due to: an electronic instrumentation effect; a random accidental correlation; or a rare version of one of the well known 'local' physical phenomena. The evidence we used to rule out the first possibility lies both within the event itself and in the other tape records acquired in the hours preceding and following this event. A normal record containing one pulse occurred 30 s before the event and another one 9 s after the event.

We observe about 300 twofold coincidences per day and about four threefolds per day within our 0.1 s time resolution. These rates are in good agreement with an accidental prediction based on our 'singles' rates. From these rates we would expect

Table 1 Details of the burst data

Burst	Counter					
	1	2	3	4	5	7
1	0.10 μs 0.60 μs	0.15 μs	0.05 μs	0.05 μs 0.45 μs 1.00 μs	0.15 μs	0.00 μs
2	639.65 μs	639.65 μs 640.00 μs 640.40 μs All readout scalars filled	639.60 μs			639.70 μs
3	1607.60 μs All readout scalars filled		1607.50 μs 1607.80 μs All readout scalars filled	1607.60 μs All readout scalars filled	1607.65 μs 1608.00 μs	1607.45 μs 1608.10 μs All readout scalars filled
4					2535.7 μs All readout scalars filled	

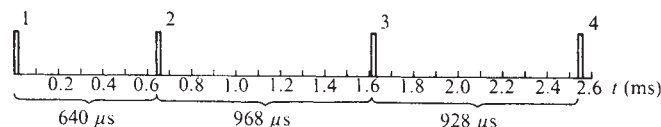


Fig. 2 Time distribution of narrow bursts of observed pulses.

a 24-fold accidental correlation within $0.1 \text{ s} \approx 10^{-40}$ times per second. A 3-ms correlation requirement would reduce this rate considerably.

A higher rate can be obtained by considering the probability of three large angle muons traversing the apparatus within 3 ms. We observe about 7.5 vertical muons per day traversing each detector and see a large angle muon going through two detectors about once in two days. From these numbers we predict about one muon traversing all our detectors once a year or three muons within 3 ms $\approx 10^{-30}$ times per second. Of course, this mechanism would not account for the multi-pulse structure of the bursts, nor for the small pulse heights associated with our pulses.

Extensive cosmic-ray showers can have a spread in time of arrival spread of $\sim 150 \text{ ns}$ (ref. 4). This spread is consistent with the time distribution of pulses within one of our bursts. We observe about one cosmic ray shower per week at our apparatus but very few of them are spatially spread over more than one detector. In 3 months of observation we have seen only one shower involving three detectors and none that spans more than three detectors. Clearly the frequency of showers that span all our detectors $\leq 10^{-7} \text{ s}^{-1}$ and so the probability of having three such showers within 3 ms $\leq 10^{-26} \text{ s}^{-1}$. This process is also in disagreement with the small observed pulse heights.

We have considered several other correlation chains including those involving local radioactivity, and have found similar low occurrence probabilities. We are thus led to consider the 24 counts as correlated and probably due to ν_e interactions. Using 24 counts in 12 m^3 of H_2O we obtain a lower limit ν_e

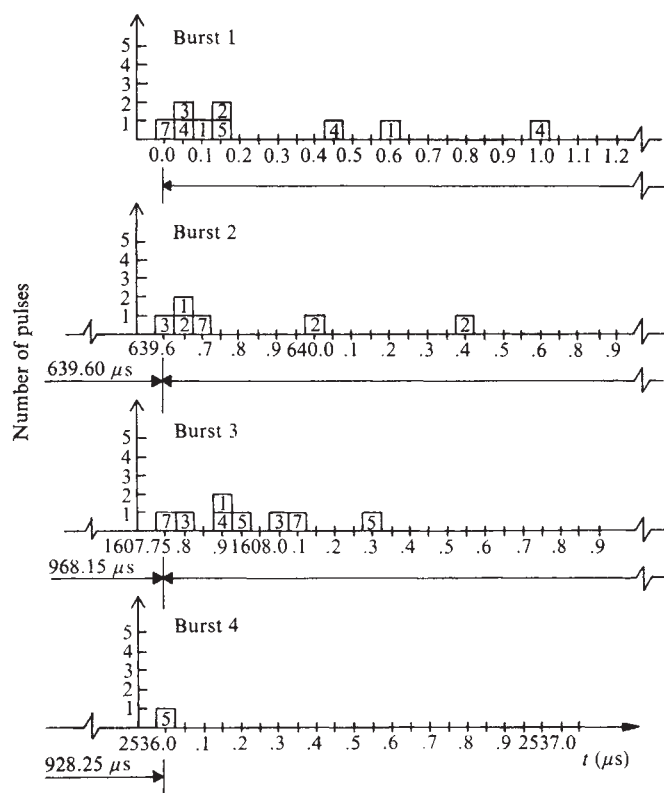


Fig. 3 Time distribution of counter pulses within each burst. The number within each box indicates the number of the counter giving rise to that pulse.

to the $\bar{\nu}_e$ flux of $1.5 \times 10^{11} (50 \text{ MeV}/E_{\bar{\nu}_e})^2 \bar{\nu}_e/\text{cm}^2$, or $1.2 \times 10^7 \text{ erg cm}^{-2}$ for 50 MeV anti neutrinos.

No additional direct experimental information is available. We can, however, speculate on possible sources such as the gravitational collapse of stars. For such a source near the galactic centre ($d \approx 3 \times 10^{22} \text{ cm}$) we find a source flux of $1.4 \times 10^{63} \text{ erg}$ (for $E_{\bar{\nu}_e} = 50 \text{ MeV}$) which is consistent with the prediction of Zel'dovich and Guseinov⁵ for the emission of neutrino energy by a collapsing star. Various people⁶⁻¹⁰ have pointed out that a collapsed star is likely to 'bounce' a number of times and that the bouncing periodicity is $\approx 1 \text{ ms}$. The narrowness of the pulses of emitted radiation may be a consequence of the fact that the collapsed star emits antineutrinos mainly during the period its density is maximum, at the minimum of its bounce.

If we assume that the energy distribution of the $\bar{\nu}_e$ has a width comparable to $E_{\bar{\nu}_e}$ then from the observed narrow pulse width we can place an upper limit on the mass of $\bar{\nu}_e$ of $10^{-2} \text{ eV}/c^2$.

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Brookhaven solar neutrino detector and collapsing stars

THE Brookhaven solar neutrino detector has been in continuous operation in its present form since 1970. The basis of the detection method is the inverse beta decay reaction on ^{37}Cl to produce ^{37}Ar ($t_{1/2} = 35.1 \text{ d}$) which can be detected at a sensitivity level of 10 to 20 atoms. The detector, consisting of 380,000 l of C_2Cl_4 , is described in detail elsewhere (refs 1-3 and references therein). Measurements with this detection system establish¹⁻³ an upper limit for solar neutrinos of 1 SNU (1 SNU $\equiv 10^{-36}$ captures/ ^{37}Cl atom-s). This limit is in substantial disagreement

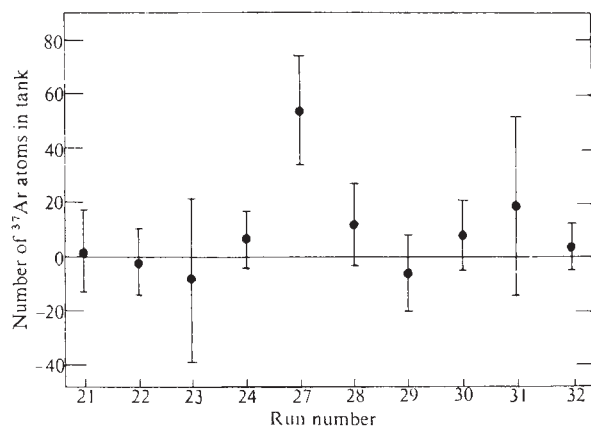


Fig. 1 The number of ^{37}Ar atoms found in the C_2Cl_4 tank at the end of each helium sweep is plotted for each run. A cosmic-ray background correction has been subtracted. The error bars shown are purely illustrative and are based on the square roots of the small counted numbers.

with accepted solar model calculations⁴ which predict a result of 5.6 SNU (ref. 5). The significance of this discrepancy has been recently analysed in detail⁶; this number also represents a base line for comparison with other possible sources of high energy neutrinos^{3,7}, in particular those produced by collapsing stars.

On January 4, 1974 a possible collapsing star event was observed by the University of Pennsylvania water Cerenkov antineutrino detector located next to the Brookhaven detector in the Homestake Gold Mine⁸. The C_2Cl_4 tank was swept as soon as possible after this event in an attempt to detect an associated neutrino pulse. The result of this search was negative and can be used to set a limit on the ratio of antineutrinos to neutrinos escaping from a collapsing star—if that is indeed the source of the event.

Figure 1 shows the experimental results for ten runs with the Brookhaven neutrino detector in its present form. The cosmic ray correction⁹ amounts to a subtraction of about 3 to 4 atoms per run. Of the ten measurements to date, only one, run 27 (July 7 to November 5, 1972) seems to contain any measurable ^{37}Ar , in contradiction with the other runs. A simple statistical test, Chauvenet's criteria, suggests that the point is abnormal and for that reason it is not included in the solar neutrino calculation. A more sophisticated statistical analysis of the data¹⁰ also suggests that the measurement is anomalous. The number of ^{37}Ar atoms in the tank at the time of purging run 27 (November 5, 1972) was 58 ± 20 , compared to an average of 8 ± 7 ^{37}Ar atoms for all other runs. The number of ^{37}Ar atoms in the tank produced by cosmic rays is estimated⁹ to be 4 ± 2 . Apart from statistical considerations, the data for run 27 show a rather convincing signature for ^{37}Ar Auger electron decays, agreeing well in energy, pulse rise time and half life. The possibility that this ^{37}Ar was introduced by some type of contamination was carefully considered and found to be extremely unlikely. The various contaminations considered were: (1) the activity of ^{37}Ar in atmospheric argon from underground nuclear bomb testing and the amount of air argon introduced in the sample; (2) ^{37}Ar in the argon added directly to the counter filling; and (3) ^{37}Ar remaining in the counter and the filling line from previous measurements. The amount of ^{37}Ar found was about 10 to 20 times the normal cosmic ray rate. Pallister and Wolfendale¹⁰ examined the possibility that this amount was simply due to a large fluctuation in the cosmic-ray interaction rate and concluded this was also extremely improbable. A solar flare occurred on August 4, 1972 which was the largest ever observed. But this is an unlikely source of neutrinos. The experimental facility is sufficiently deep underground to be totally unaffected by the relatively low energy charged particle flux. On September 2-11, 1972 a large radio outburst was recorded from Cygnus X-3, but again this is

generally believed to be an unlikely source of neutrinos. A γ -ray pulse of unusually sharp rise time was observed by the Vela satellites on November 1, 1972 (ref. 11). The energy flux in this pulse was very small and it is difficult to say whether or not this event is related to the excess in run 27. The University of Pennsylvania detector was not yet operational at the time and so it is not possible to say whether or not the excess was related to a collapsing star.

Run 32 was collected on January 25, 1974 about three weeks after the event detected by the University of Pennsylvania water Cerenkov detector. The result, with cosmic-ray background subtracted, is 4.5 ± 9 ^{37}Ar atoms in the tank at the time of the sweep. This is in good agreement with eight other runs which give an average of 4 ± 7 ^{37}Ar atoms (Fig. 1). If a decay correction is included, a 1σ limit of ≤ 21 atoms of ^{37}Ar produced on January 4, 1974 is obtained. To compare this number with the University of Pennsylvania result, a knowledge of the relative sensitivity of the two detectors is required. The cross sections for the two reactions were calculated¹² using the best available values for the matrix elements and Fermi function. A calculation of the ratio of the neutrino reaction (σ_{37}) to antineutrino reaction (σ_1) at various particle energies shows that the ratio is about 1.1 at 20 MeV ($\sigma_{37} = 3.1 \times 10^{-41} \text{ cm}^2$), the threshold for the Cerenkov detector, and approaches an asymptotic value of 1.6 at high energy. At very high energies, the ratio would presumably start to drop again due to nucleon recoil effects, but that is outside of the energy range of interest for collapsing stars. Since the chlorine detector contains about 2.6 times as many usable target atoms as the Cerenkov detector, the efficiency of the chlorine detector is 2.9 to 4.2 times that of the Cerenkov detector for equal particle fluxes and energies in the energy range of interest. Combining this result with the 24 counts seen in the Cerenkov detector, a minimum of 70 atoms ^{37}Ar should have been produced in the chlorine detector for equal particle fluxes and energies. The antineutrino to neutrino flux ratio is $\geq 4:1$ assuming a typical particle energy of 50 MeV ($\sigma_{37} = 2.9 \times 10^{-40} \text{ cm}^2$). The pulse recording system used in the water Cerenkov system was only capable of recording a maximum of 24 pulses during the time period of interest. There is therefore a large uncertainty in the true size of the event. The corresponding flux ratio may in fact be significantly larger since the chlorine detector integrates the entire event.

There are several restrictive conditions that must be satisfied if the January 4 event is to be interpreted as a collapsing star. First, the expected time duration for a pulse of neutrinos to diffuse out of the central regions of a star is $\sim (R/c) (R/\lambda)$, where R is the characteristic size of the collapsing star ($\sim 10^{6.5} \text{ cm}$) and λ is the average mean free path of the antineutrinos. In order for there to be significantly more antineutrinos than neutrinos leaking out, (R/λ) must be large. The characteristic time for changes in the physical conditions is of the order of the dynamical time and is also long, $\sim 1 \text{ ms}$. So the main pulse width should be $> 10^3 \mu\text{s}$ instead of the observed width $\sim 1 \mu\text{s}$. The main pulse should have (see equation (14) of ref. 14) $\geq (R/3)$ times (approximately 100 times) as many neutrinos and antineutrinos as in the prompt burst. This increases the total estimate of energy in neutrinos and antineutrinos for the model of ref. 12 to ~ 0.3 to $1 M_{\odot} c^2 \times (\text{distance}/10 \text{ kpc})^2$ if a fairly flat spectrum of energies is assumed.

The failure to observe neutrinos associated with the event of January 4 is also a serious constraint on possible interpretations in terms of collapsing stars. Under most astrophysical circumstances one would expect approximately equal numbers of neutrinos and antineutrinos whereas the combined observations imply an antineutrino flux more than four times the upper limit on the neutrino flux. The University of Pennsylvania water Cerenkov detector is not sensitive to neutrinos below $\sim 20 \text{ MeV}$ whereas the Brookhaven ^{37}Cl detector has considerable sensitivity in this region¹². If one interprets the event of January 4 as being produced by antineutrinos then one must say the flux was primarily antineutrinos more energetic than

20 MeV. But one numerical calculation of neutrino transport in a collapsing star yields¹⁴ spectra with a peak at about 10 MeV.

Also the initial stages of the collapse of a white dwarf to form a neutron star would produce predominantly neutrinos¹⁴ through electron capture, transforming protons into neutrons, and would yield very few antineutrinos (provided the star is made out of matter, not antimatter).

The likely production processes at higher temperatures and densities yield equal numbers of neutrinos and antineutrinos; this is true for all processes based on the interaction $e^+ + e^- \rightarrow \nu_e + \bar{\nu}_e$ and for two nucleon collisions ($n + n \rightarrow p + n + e^- + \bar{\nu}_e$; $p + n + e^- \rightarrow n + n + \nu_e$). In nondegenerate, high temperature regimes ($T_{20\text{MeV}}^3 \rho^{-1} \gtrsim 10^3$) such as might occur in shock waves formed by supernovae¹⁵, the principal opacity would be due to lepton-lepton scattering¹⁶ ($\nu_e + e^- \rightarrow \nu'_e + e^-$, $\bar{\nu}_e + e^- \rightarrow \bar{\nu}'_e + e^-$ and so on) which leads to equal opacities for neutrinos and antineutrinos if, as is true under such conditions, the number of electron-positron pairs in equilibrium with the radiation field is large compared to the number of atomic electrons. (The maximum difference between ν -e and $\bar{\nu}$ -e cross sections for any intermediate vector boson theory with μ -e symmetry is a factor of three¹⁸ and for the Weinberg-Salam model with $\sin^2\theta_w = 0.3$ to 0.4 is a factor of two) (compare ref. 19).

Most astrophysical events are expected to give rise to at least as many neutrinos as antineutrinos. Moreover, accompanying the prompt antineutrinos to which the University of Pennsylvania detector is sensitive one would expect a much larger number (on a longer time scale) of lower energy neutrinos (and antineutrinos) to which the Brookhaven detector is sensitive. The absence of detectable neutrinos is puzzling if the event of January 4 was caused by antineutrinos from a collapsing star.

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Possible explanations of the 'high' number of counts in the solar neutrino experiment

EVANS *et al.* (see also refs 2, 3) have presented data on the rate of production of ³⁷Ar with the Brookhaven Solar Neutrino Detector; two facts stand out: the overall mean rate is low compared with expectation⁴, and one run, No. 27, may be significantly higher than the remainder. The present note is primarily concerned with the statistical significance and explanation of this high rate. The calculation of the rates and uncertainties is not a simple procedure and it is important to have an independent determination; with the cooperation of the authors, we have made our own analysis using their basic data (β -ray counter measurements of the β -activity of the abstracted ³⁷Ar over successive periods of ~ 30 d), for the important run 27.

Several methods have been adopted to determine the rate R: I, the maximum likelihood method; II, a similar method used in the Case-Wits-Irvine Experiment by Meyer⁵, and III, a graphical method in which isoprobability contours of the observed configuration of counts are calculated, and cuts through them taken and the resulting, normalised differential probability curves integrated to determine the probability that the true number of atoms at the end of exposure was less than some particular value N_0 :

$$P(<N_0) = \int_0^{N_0} \int_0^\infty P(N_0, b) dPdb$$

where b is the small but finite counter background. The values of N_0 at which $P(<N_0)$ are 16% and 84% correspond to the 'error' in the mean rate. All the calculations gave R_{27} as 1.18 ³⁷Ar per day (some of the residual difference with the Davis estimate of 1.24 ± 0.43 ³⁷Ar per day arises from small differences in input data). Methods I and II gave the standard deviation of the rate ($\sigma=0.43$), but because of the skew nature of the probability distribution this bears no simple relationship to the probability that the mean rate is within the above bounds. Method III gave errors of -0.31 and $+0.41$ ³⁷Ar

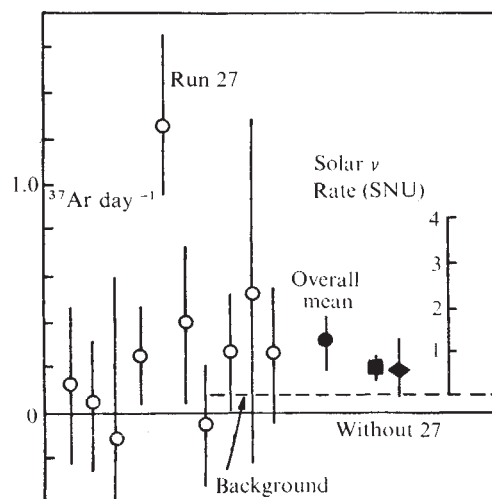


Fig. 1 Results of the solar ν experiment, after Evans *et al.*¹. The errors on individual run rates (open circles) quoted for Run 27 come from the present work, the rest from ref. 1. The mean values are those derived by us.

Table 1 χ^2 and probabilities for various situations

Adopted 'true' value	Data Examined	χ^2	$p(\chi^2)$
Overall mean	All data	11.7 (14.0, 21.8)	16% (8%, 0.5%)
	R_{27} excluded	2.9 (4.0, 11.0)	90% (78%, 14%)
Mean excluding R_{27}	All data	13.2 (15.0, 22.2)	10% (6%, 0.5%)
	R_{27} excluded	1.8 (3.6, 10.7)	97% (82%, 15%)
Expected background	All data	16.3 (18.8, 30.1)	4% (1.5%, 0.02%)
	R_{27} excluded	2.6 (5.1, 16.3)	92% (66%, 2.3%)

The figures in parenthesis refer to the situation where the errors for runs other than No. 27 are multiplied by factors of 0.7 and 0.4 respectively. It is likely that the true situation gives χ^2 probabilities intermediate between the values in parenthesis.

per day, an increase of 0.12 on the lower limit estimated by Davis, lending further support to the suggestion of the anomalous character of run 27.

To establish the significance of the high value of R_{27} , various χ^2 tests have been performed using the data shown in Fig. 2, including and excluding run 27, and using several reasonable estimates of the true mean rate. The results shown in the table indicate that there is some evidence of an excess in R_{27} . But as was pointed out by Evans *et al.*¹, there is a suggestion that the quoted errors (other than R_{27}) are too large; this is borne out by the high probability (90%) for the appropriate conditions.

An alternative approach is to examine the errors and the dispersion of the mean rates about their mean. Excluding run 27, the dispersion error α , is 0.05 which is much smaller than the value expected ($\approx \langle \sigma \rangle / \sqrt{n} \approx 0.12$, where $\langle \sigma \rangle$ is the median of the individual errors), on the basis of the errors of the individual rates. This confirms that the quoted errors are probably overestimates. In view of the uncertainty of the individual errors, the χ^2 probabilities have also been calculated with the errors on runs other than 27 reduced by factors of 0.7 (the ratio of 'our' error on R_{27} and the quoted error) and 0.4 (the ratio of the dispersion and 'expected' errors) respectively. The results (see the Table 1) show that the significance of the excess is considerably enhanced. In particular, if the smallest errors are adopted then Run 27 is almost certainly anomalously high. More data over the next year or so will surely enable a more definite conclusion to be arrived at.

It is interesting that if the dispersion errors are correct, then, neglecting R_{27} , the upper limit on the residual solar neutrino rate is only 0.9 solar neutrino units (SNU), and the solar model calculations are in disarray⁴. Inclusion of run 27 raises the upper limit to ~ 1.9 SNU and the discrepancies are not quite as severe.

Assuming R_{27} is anomalously high, various possibilities arise:

- (i) The presence of an unusual, non-Poissonian, increase in background.
- (ii) The effect of a large solar flare (August 4, 1972).
- (iii) The arrival of neutrinos from a cosmic source.

The background is composed of two parts:

(a) Cosmic ray neutrinos produced in the atmosphere contribute $\sim 30\%$ to the background and are extremely unlikely to have large fluctuations over the integrated time scale of the detector.

(b) The contribution from protons, 'knocked on' by fast cosmic ray muons to the reaction $^{37}\text{Cl} (p, n) ^{37}\text{Ar}$. Knowledge of the muon spectrum, nuclear cross section, and yield of ^{37}Ar atoms per interaction ($\propto$ (Energy transfer)^{0.7}, following Wolfendale *et al.*⁶) enables the contribution to the background from rare events producing many ^{37}Ar atoms (when large fluctuations may occur) to be determined. This contribution is very small which implies that the background fluctuation should be strictly Poissonian with a total mean value of 0.09 ± 0.04 ^{37}Ar atoms per day.

Solar flare. Evans *et al.* have rightly implied that the muons formed from protons in the solar flare have insufficient energy to penetrate to tank depth. More important is the

increase in the neutrino flux from the low energy muons decaying high in the atmosphere but their contribution is much too small to provide the excess in R_{27} . (We thank C. J. Hatton for comments on this point).

Cosmic source. Evans *et al.* have sought an explanation of the neutrino excess in terms of cosmic sources (the γ -ray pulse event of November 1, 1972)⁷. The unusually short pulse width (~ 0.1 s) is of the same order as the expected collapse time of a star to the neutron stage when a large neutrino burst is expected⁸. The probability of the solar neutrino detector recording a signal of more than 50 counts per run from collapsing stars has been considered by us⁹ and shown to lie in the range 10^{-1} – 10^{-2} if all stars of mass $> 1.5 M_{\odot}$ collapse. Such a probability does not disagree with run 27 corresponding to a collapsing star (one ν -burst in 10 runs), and assuming R_{27} is anomalously high, we would regard this explanation as perhaps the most probable.

Finally, regarding the exciting result reported by Lande¹⁰ we point out that events of this type, if genuine, are detectable by extensive air shower arrays, when the positrons released in the detector simulate a shower of completely flat lateral distribution. Comparing the threshold sensitivity of the two major arrays, 60 MeV m⁻³ μs^{-1} water at Haverah Park and 80 MeV m⁻³ μs^{-1} at Sydney, with the energy deposition observed by Lande (15–75 MeV m⁻³ μs^{-1}) both figures are seen to be in the region of observed energy deposition. Neither array observed a coincidence with the 'Penn' event but this is understandable if the energy deposition was near 15 MeV m⁻³ μs^{-1} .

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Anomalous mercury in near-bottom water of a Mid-Atlantic Rift valley

MERCURY analysis of over 1,000 water samples from GEOSECS stations in the western Atlantic indicated extremely low mercury concentrations (2–40 ng l⁻¹) in much of the ocean between 15°N and 35°S, and identified a large core of water of relatively high concentration (80–400 ng l⁻¹) between 25°N and 50°N. These increased concentrations may result from the entrainment of Hg which diffuses from the active spreading centre of the Mid-Atlantic Ridge, by Mediterranean water which flows over

Table 1 Mercury in samples of seawater from the FAMOUS area

Sample number	Hg (ng l ⁻¹)	NaCl (‰)	F : Cl (× 10 ⁵)	O ₂ (ml l ⁻¹)
2	1,380 ± 90	35.024	6.66	—
10	1,190 ± 90	35.020	6.64	6.11
11	1,420 ± 90	35.012	6.66	6.10
13	1,330 ± 90	35.031	6.69	6.04
15	1,280 ± 90	35.014	6.70	—
16	980 ± 90	34.966	6.64	—
17	900 ± 90	34.985	6.69	6.09
28	1,180 ± 90	35.017	6.70	5.91
29	1,000 ± 90	35.033	6.62	—
59	920 ± 90	35.018	6.74	5.94
61	900 ± 90	35.015	6.70	5.92
66	1,030 ± 90	35.014	6.64	6.01
67	870 ± 90	35.014	6.70	6.00
68	1,130 ± 90	35.012	6.76	6.03
69	880 ± 90	35.012	6.74	5.99
Mean	1,090	35.012	6.69	6.01
s.d. of mean	± 193	± 0.017	± 0.04	± 0.07

the ridge into the western basin of the Atlantic Ocean. Mercury concentrations are as high as 1,370 ng l⁻¹ at a depth of 2,435 m at GEOSECS station 27 (42°00'N, 41°59'W), (ref. 1).

Bostrom and Peterson² have described sediments enriched in Fe, Mn, B, As, Cd, V and Cr in areas of high heat flow on the East Pacific Rise. They suggested that these sediments have incorporated precipitates of the volcanic emanations from active ridge crests. White³ examined the composition of hot spring waters and suggested that trace elements such as Ba, W, Zn, Hg and Cu could also be enriched in the products of outgassing. Mercury in Japanese hot spring waters has been reported⁵ to range from 100–30,000 ng l⁻¹.

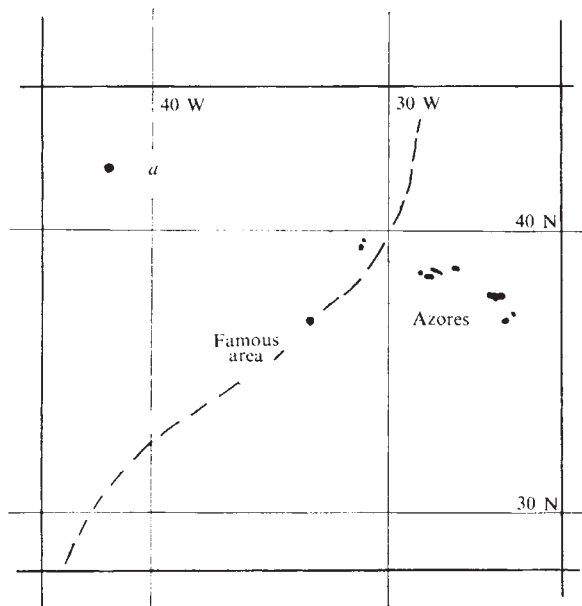


Fig. 1 Location of the FAMOUS Area. Dashed line, Mid-Atlantic Ridge; a, GEOSECS Station 27.

During one phase of Operation FAMOUS (French-American Mid-Ocean Undersea Study), we took water samples from near the bottom of a rift valley at 36°49'N, 33°16'W (Fig. 1), at a depth of 3,200 m. The iron:manganese ratio of the samples averaged 9.2, close to the average value of 10 observed for ocean water (unpublished data from this laboratory). The 15 samples were analysed for mercury using the method of Carr *et al.*⁵, with a best estimate of error ± 90 ng l⁻¹. The analysis showed a mean value of 1,090 ng l⁻¹, with a standard deviation of the mean of 193 ng l⁻¹ (Table 1). The difference

between samples is significant. All the samples seem to have come from the same water parcel, as is indicated by the uniformity of values for salinity, dissolved oxygen (Table 1), and by the temperature: 3.97 ± 0.03°C. The F:Cl ratio was that of normal sea water. These data support the hypothesis that mercury is introduced into the sea water column at active spreading centres.

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Three dimensional image reconstruction on an extended field—a fast, stable algorithm

WE describe an improved algorithm (EFIRT) for solving iteratively the linear equations relating a three-dimensional density to a given set of its projections, when the unknown density is expressed as samples on a grid. The need to solve such equations arises in electron microscopy, medical radiography, radio and X-ray astronomy and other fields. The projection equations comprise a very large, sparse and often under-determined set. If the number of unknowns is not too large, the equations may be solved by a least squares procedure, using filtering to combat the ill-conditioning¹. The number of unknowns is often too great for such a procedure to be computationally feasible and iterative techniques^{2,3} must be used. Alternatively the Fourier transform provides a stable and computationally efficient means of solution¹. Sometimes use of the Fourier transform is not convenient; for example, if point rather than plane projections are given or if the projection data are weighted by a non-uniform but linear response of the measuring device. Such problems are much more readily expressed in terms of projection equations and the development of stable methods for their solution is therefore important.

If the observations contain noise the set of projection equations will in general be inconsistent. This is aggravated by the approximations involved in representing the continuous density in the object by a discrete set of samples. Existing iterative methods of solution are unstable in these circumstances, though the SIRT algorithm has proved much better than the ART algorithm^{3,4}. The Extended Field Iterative Reconstruction Technique (EFIRT) presented here attempts to overcome this instability by performing the reconstruction on an extended field which is larger than the object. Unlike any iterative technique so far described, this allows errors and inconsistencies to propagate outside the object domain in the reconstruction. The extended field effectively provides a 'rubbish bin' for a large proportion of the noise, so that it does not spoil the region of interest. The rationale for this approach comes from a Fourier analysis of the problem.

We take the simple case of data collected by tilts about a single axis, so that reconstruction reduces to solving for a two-dimensional section from a set of line projections. We denote the unknown sampled density values in the object by

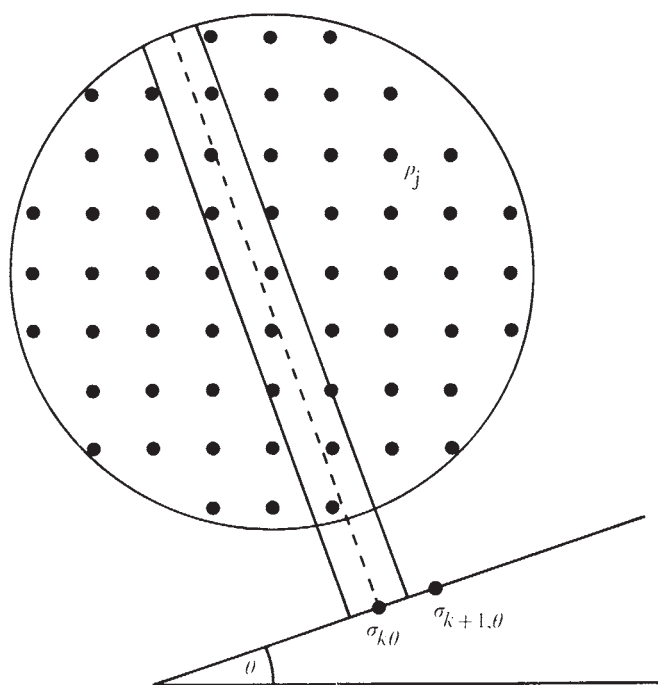


Fig. 1 Densities ρ_j of the object are reconstructed from projection data $\sigma_{k\theta}$. The diagram shows the k^{th} ray for the projection at angle θ , which is used in calculating the ray sum $R_{k\theta}^{(q)}$ of the current approximation $\rho_j^{(q)}$. The number of sample points ρ_j included in the ray is $N_{k\theta}$ and the length of the ray within the circular object domain is $L_{k\theta}$. The width of the ray is equal to the spacing of the sampling lattice for the ρ_j .

ρ_j and the total set of sampled projected densities by σ_i (Fig. 1). The two sets of quantities are related by the projection equation

$$\sigma_i = \sum_j p_{ij} \rho_j$$

If a simple ray sum approximation is made, the elements p_{ij} of the projection matrix are 0 or 1. If some form of interpolation is used to express the density between sampled values ρ_j , the p_{ij} are fractions between 0 and 1. We assume the former simple approximation here.

Initially all the density values $\rho_j^{(0)}$ are set equal to the mean density level in the object, which can be estimated from the projections. We then generate successive approximations $\rho_j^{(q)}$ to the density using the formula³

$$\rho_j^{(q+1)} = \rho_j^{(q)} + (\sigma_{k\theta}/L_{k\theta} - R_{k\theta}^{(q)}/N_{k\theta}) \quad (1)$$

Here $\sigma_{k\theta}$ represents the observed projected density at point k in the projection at angle θ , $R_{k\theta}^{(q)}$ represents the ray sum of the current approximation $\rho_j^{(q)}$ for the k^{th} ray in the projection at angle θ , and $L_{k\theta}$, $N_{k\theta}$ are respectively the length of the ray lying in the object and the number of sample points ρ_j within it. For a given j in equation (1), only one point k is included for each θ (Fig. 1). It is used for all points in the first projection, then the second and so on until all projections have been used, completing one cycle of iteration. Further cycles of iteration can then be made. The use of equation (1) makes each ray sum $R_{k\theta}^{(q+1)}$ for the approximation $\rho_j^{(q+1)}$ equal to the observed projected density for the particular projection θ most recently used.

The overall size of the object can be estimated from the given projections and we assume that it is contained within a circle of radius a . If it is clear from the projections that the object is not isometric, some other choice of domain can be made. The essential point of EFIRT is that we apply equation (1) not only to points within the object domain of radius a but to all points within a circle of radius b , say, where $b > a$. We thus have an annular region outside the object in which errors can

propagate. Initially the density $\rho_j^{(0)}$ is set to the mean density within the estimated object domain and to zero in the annular region. In the iteration, those projected density values, $\sigma_{k\theta}$, which correspond to rays passing only through the annular region and not through the object, are taken to be zero.

The density in the annular region arises from noise, inconsistencies and possibly 'fringe effects' due to the limited resolution obtainable from the finite set of projections and it need not therefore be positive. Indeed, in general it will be equally positive and negative. So the algorithm (1) must not be constrained to give positive density in the annular region, though the constraint can be applied within the object itself. Clearly multiplicative algorithms, which correct each ray sum by multiplicative rather than additive scaling, are not applicable.

With noisy data the use of this extended field leads to a great improvement in both the stability of the iteration and the quality of the resulting reconstruction. From numerical experiments with a known test object and its projections, it is possible to tell how close the reconstruction is to the true object density after each iteration. We express the root mean square difference in density between the reconstruction and the object as a fraction, δ , of the standard deviation of the density within the object. Thus normalised, δ is a measure of how much closer to the object is the reconstruction, than would be a uniform distribution equal in value to the mean of the density in the object.

The test object (Fig. 2) consists of a set of uniform discs³, whose projections are easy to compute analytically. We have taken 25 equally spaced projections ($0 < \theta < \pi$). The true projections are made noisy by adding at each point a pseudo-random number uniformly distributed in the interval $(-0.1\bar{\sigma}, 0.1\bar{\sigma})$, where $\bar{\sigma}$ is the mean projected density. In a second example each projected density is multiplied by a pseudo-random number from a Gaussian distribution with mean 1.0 and standard deviation 0.1. The course of the iteration is plotted in Fig. 3 for the two examples. We have held constant the radius a of the object at 12 sample steps and increased the radius b of the total field giving an increasing annular region for error propagation. Shannon sampling corresponding to the Fourier cutoff $R_{max} = (25)/(\pi 2a)$ appropriate to 25 views¹ would give about 8 samples in the radius of the object, so the

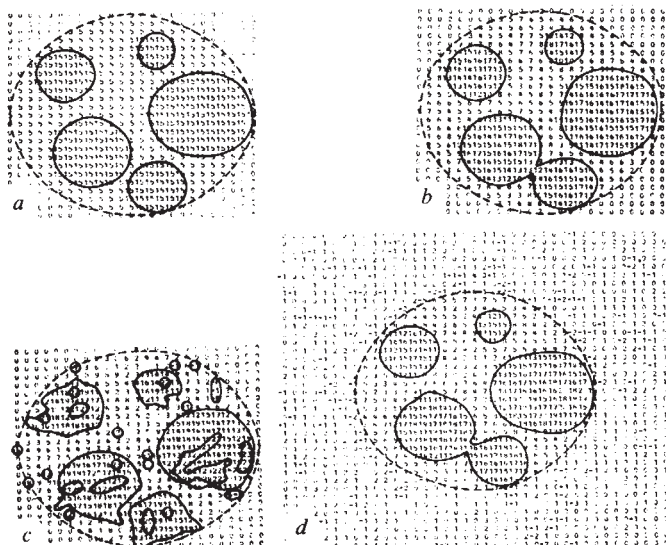


Fig. 2 *a*, Model structure, consisting of a set of circular discs of uniform density, used for testing the algorithm. The printout is compressed by 20% in the vertical direction giving a distorted appearance. The reconstructions were made from 25 equally spaced projections to which 10% uniform noise has been added. *b*, Reconstruction by R -weighted back-projection. *c*, Reconstruction after 1 cycle of iteration with a non-extended field ($b/a = 12/12$). *d*, Reconstruction after 1 cycle of iteration with EFIRT ($b/a = 24/12$). Only part of the extended field is shown.

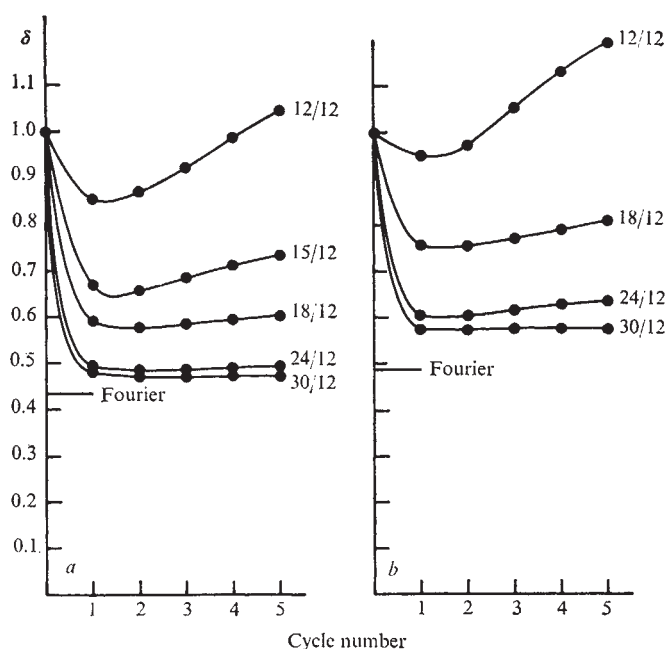


Fig. 3 The discrepancy δ (see text) plotted against the number of cycles of iteration of EFIRT, for the reconstruction of the object shown in Fig. 2a from 25 projections containing noise. The values of δ are computed using only those densities within the circle of radius a . Each curve is for a different size of extended field as indicated by the value of b/a appended. Also shown is the value of δ for a reconstruction made by a Fourier method, as described in the text. In *a*, 10% uniform noise was added to the projections. In *b*, 10% Gaussian multiplicative noise was included.

sampling chosen is fairly generous. Increasing the frequency of sampling to 18 in the radius produced only a slight improvement. Presumably this is because the total number of grid points in the object domain is already about equal to the number of recoverable parameters set by the number of views⁶.

When $b/a = 12/12$ and there is no annular region (so that we are essentially using the ART algorithm²), the value of δ falls slightly at the first iteration but thereafter increases rapidly giving rise to a reconstruction which bears little resemblance to the original. The effect is of an increasing 'peppering' of the reconstruction (Fig. 2c). But as the value of b is increased, the value of δ after the first cycle falls dramatically (Fig. 3). Even a small extension of the field produces a significant effect. Figure 2d shows the improved reconstruction obtained after one cycle when $b/a = 24/12$, again for the case of additive noise. There is now density in the annular region outside the object. Furthermore after the steep fall at the first cycle of iteration, δ tends to become more and more constant in successive cycles as b is increased (Fig. 3). But increasing b beyond a certain size produces little extra improvement. A similar behaviour was observed with other test objects, although the effects were less marked when fewer views were used. The extended field algorithm is so rapidly convergent that only one complete cycle of iteration is required, compared with about 15 cycles when using SIRT (ref. 3). In spite of extra computation required when working with an extended field, the method is at least as fast as any other stable iterative method.

Also indicated in Fig. 3 are the values of δ obtained when the reconstruction is done by a Fourier transform method, in this case R -weighted back-projection^{6,7}. We perform one-dimensional transforms on the projections and weight^{6,7} the frequency components by transform radius R out to a radius R_{max} and then by $(2R_{max} - R)$ out to a radius $2R_{max}$, where R_{max} is set by the number of views and the noise level. We then inversely transform to produce a set of modified R -weighted projections. The reconstruction is then made by back-projecting these modified projections. The reconstruction from the data containing additive noise is shown in Fig. 2b. Although the values

of δ obtained by this Fourier method are lower than the best δ achieved by EFIRT for both types of noise, the differences are probably not significant since the projection equations used in EFIRT were of the simplest type, without interpolation.

Fourier analysis shows that besides the recoverable components giving density inside the object, there are a further set of recoverable components which build density outside the object^{1,8}. In the Fourier method¹, these external components are not explicitly calculated, but are implicit and serve to carry a certain part of the noise. With perfect data from a spatially limited object, the external components will be zero and the set of projections can be completely interpreted, to a resolution set by the number of projections, in terms of the density within the object. Once there is noise in the projections, the external components will be non-zero and the projections will no longer be completely interpretable in terms of density within the object. In these circumstances, the projection equations set up for the object domain only, become inconsistent. But by taking an extended field, which admits the possibility of building density outside the object, the equations become consistent and the convergence of the iterative procedure is stable and rapid. Similar rapid and stable convergence was observed² when the ART algorithm was applied to pseudo projection data (when the ray sums $R_{k\theta}$ of the object are used in place of the true projected densities $\sigma_{k\theta}$), in which case the projection equations are again perfectly consistent, but this situation does not correspond to actual reconstruction from true projections.

It is clear from Fig. 3, that in these examples at least, most of the improvement to be gained by extending the field has already been obtained when the diameter of the total field is twice that of the object. Further increases in the size of the field make only small changes to δ . If all the external components were of equal weight, the annulus between a and $2a$ can be calculated from Fourier theory to contain about 75% of the external squared density. It seems that in the examples considered here an even larger proportion of the total external density is contained in the annulus between a and $2a$. The optimal size of the extended field will almost certainly depend on the type of object being reconstructed and on the nature and amount of noise in the projections. It is clear, however, that the extension of the field has a profound effect on the behaviour of the iterative procedure, and, contrary to what might have been expected, the prescription of the boundary of the object, far from being a useful constraint in solving the equations, is actually harmful.

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Effects of sulphur content on superconductivity and intercalation in transition metal disulphides

THE nearly two-dimensional character of the group IVB and VB transition metal dichalcogenides intercalated with organic molecules¹ makes the superconducting metals logical choices for the study of superconductivity in two dimensions. Many anisotropic semiconductors could be used as electronic

devices. But scientific investigation has been restricted partly by the quality of available crystals. A number of complexes are metastable at room temperature, and others deintercalate below 150° C. Many intercalation compounds become greatly exfoliated so that measurements of single crystals become quite difficult. Other crystals exhibit little or no registry between adjacent layers. In one case—TaS₂(pyridine)_{1/2}—two phases of intercalated material have been detected. We have found that the addition of sulphur to the intercalate both orders and thermally stabilises the resulting complex. The removal of sulphur by the intercalate increases disorder and thermal instability.

A layered, transition metal dichalcogenide can be intercalated with an organic molecule (typically a Lewis base) if the dichalcogenide and organic material are brought together at a sufficiently high temperature. In the intercalation complex the organic molecule resides between layers of the dichalcogenide. In each case where intercalation occurs the supernatant organic liquid changes colour. In the case of ammonia intercalations in disulphides the colours range from dark blue to yellow and red. For other intercalations in disulphides, such as pyridine and aniline, the colour is dark brown to black. These colours have been found to be a result of sulphur in the supernatant. Sulphur forms coloured solutions with ammonia² and reacts to form long chain polysulphides with amines³. We have found that sulphur, dissolved in the various intercalates and heated to the intercalation temperature, forms a colour which is characteristic of intercalation at that temperature. Comparison of the ultraviolet with the visible spectra has verified the presence of sulphur in the supernatant.

Two experiments have been undertaken to determine the importance of the effect of sulphur on the intercalation product. First, some crystals of TaS₂ were repeatedly intercalated with ammonia until the supernatant remained clear. These crystals were then intercalated with pyridine. Second, a number of crystals were intercalated from pyridine saturated with dissolved (molten) sulphur. The results measured by the thermogravimetric analysis of deintercalation, are shown in Fig. 1. From these data it is apparent that the TaS₂(pyridine)_{1/2} intercalated once from pure pyridine is a two-phase material; that preintercalation with ammonia produces the low temperature phase; and that intercalation with excess sulphur produces the high temperature phase.

X-ray powder diffraction has shown that two layer spacings are associated with the two-phase material, 12.01 Å and 11.85 Å. The phase that deintercalates at 100° C is a single-phase with $c=2\times 11.85$ Å and that which deinter-

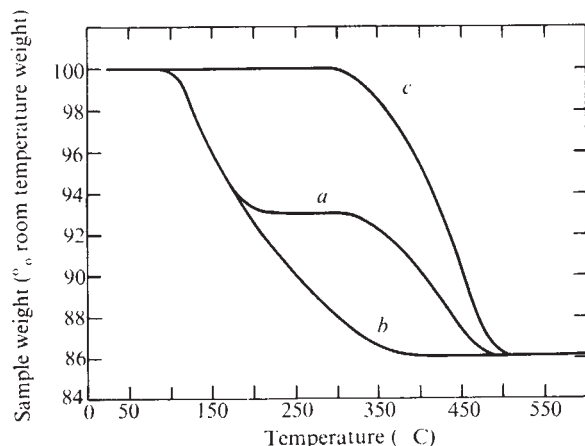


Fig. 1 Thermogravimetric analysis during deintercalation for three types of TaS₂(pyridine)_{1/2}: a, intercalated from neat pyridine; b, preintercalated with ammonia—deintercalation is complete at 100° C; c, intercalated from a mixture of sulphur and pyridine—deintercalation occurs at 300° C.

calates at 300° C is single-phase with $c=2\times 12.01$ Å. There is a qualitative difference between the diffractograms: the 12.01 Å phase has very sharp lines characteristic of a highly ordered crystal, whereas the 11.85 Å phase has broad, rather diffuse lines, giving evidence of considerable disorder. Thus, intercalation of pyridine from a saturated solution with sulphur produces a material with greater thermal stability, a larger c axis, less disorder, and little or no exfoliation.

The addition or removal of sulphur also influences the electronic and magnetic properties of intercalation complexes. Fragile, exfoliated crystals of TaS₂(pyridine)_{1/2} which are preintercalated with ammonia exhibit unusual anisotropies in the electrical properties at low temperatures⁴. These same crystals have a superconducting transition temperature that is higher than that of crystals intercalated with excess sulphur. We believe⁵ that the rise in T_c in intercalated TaS₂ is related to the suppression of the low temperature crystal distortion and to non-stoichiometry on the metal rich side.

Results which are essentially identical to these have been obtained from TaS₂ and TiS₂ intercalated with aniline. Previously, only one phase had been observed in TaS₂(aniline)_{3/4} with a c axis parameter of 18.15 Å. Intercalation from a sulphur-rich aniline solution produced an increase in c to 18.32 Å, with a corresponding increase in deintercalation temperature from 75° C to 150° C. The increase of 0.17 Å in the c axis dimension is very similar to the value of 0.16 Å obtained with pyridine. The sulphur-rich TaS₂(aniline)_{3/4} material also proved to be less exfoliated.

Intercalation normally proceeds more slowly in the presence of excess sulphur, but in the case of TaS₂(pyridine)_{1/2} the intercalation time is doubled by the addition of sulphur. The intercalation temperature seems to be correlated with the temperature at which sulphur reacts with the intercalate, thus, pyridine and sulphur do not react noticeably at room temperature, but form a dark brown-black, viscous liquid at 200° C. This reaction has been attributed to the formation of polysulphide chains on the pyridine³. A number of other intercalates have been tested (butylamine, N,N-dimethylaniline, and thiobenzamide) with similar results. For example, butylamine intercalates in TaS₂ at room temperature and there it forms a black viscous liquid with excess sulphur.

Only small amounts of sulphur are removed from the crystal during intercalation. In several ammonia intercalations the amount was too small to weigh accurately and was certainly less than 1% of the total weight of the sample. Sample intercalated in the presence of excess sulphur exhibit essentially the same increase in weight as materials intercalated with pure intercalate. Thus, any mechanism involving the transport of one sulphur atom per intercalated molecule must be rejected.

Hsu *et al.*⁶ have found that 4-vinyl pyridine can be intercalated in TaS₂, and that the thermal stability of the final product depends on the conditions of preparation. They showed that the product obtained from preintercalation with aniline is less stable than the material prepared at 140° C from 4-vinyl pyridine and *p*-methoxyphenol (a polymerisation inhibitor). They claim that the increase in stability results from the *in situ* polymerisation of the 4-vinyl pyridine. We find it more probable that they have produced two phases of the TaS₂(4-vinyl pyridine)_{1/4} complex, analogous to those reported here. The addition of sulphur has the same effect on monomeric intercalations as does polymerisation: they both increase the thermal stability.

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Thin films from liquid crystals

THE properties of thin film drawn from liquids into air have been determined in many investigations¹⁻³, because the films form suitable systems for testing the theory of colloidal stability^{4,5}. Recent results have provided an improved understanding of the state of the aqueous layer of the film^{6,7}.

Films from liquids containing surfactants display stratification in the last stages of thinning⁸, and the presence of a liquid crystal enhances foam stability⁹. These facts have illustrated the structural connections between a thin film and the repeated lamellar unit in a liquid crystal¹⁰. The lamellar liquid crystal itself has been used repeatedly as

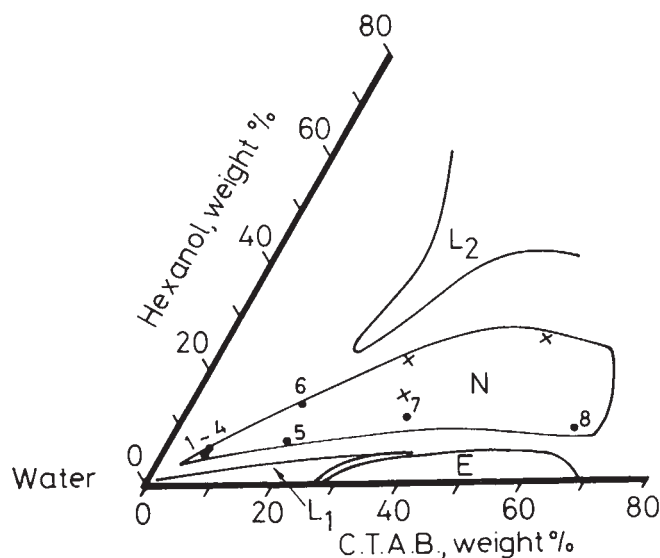


Fig. 1 The composition of the liquid crystal samples (1-8) from which a thin film was drawn. Crosses, compositions which did not give stable films; L₁, L₂, regions for isotropic liquid solutions; N, region of a 'neat phase' liquid crystal; E, region of 'middle phase' liquid crystal¹².

a model for the bimolecular layer of biological membranes. The results¹¹ on the specific influence of the distance between layers in a lamellar liquid crystal on the diffusion coefficient of sodium and chloride ions, have further increased the interest in the structural relationship between lamellar liquid crystals and thin films with a bimolecular structure.

Previous investigations of these phenomena have, however, been concerned with comparisons between structure in thin films from liquids, and the dimensions in lamellar liquid crystals¹⁰. We here report the first experiment in which a thin film has been drawn directly from a lamellar liquid crystal.

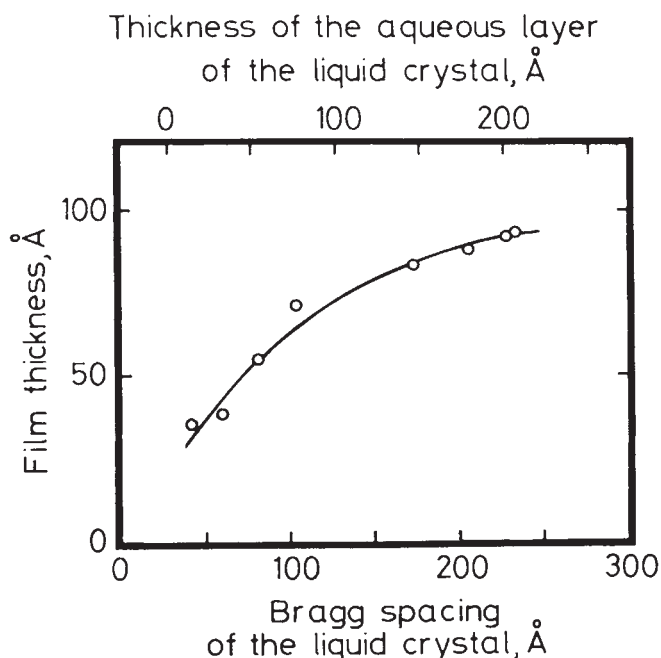


Fig. 2 Thickness of the thin film against the dimension of the lamellar unit of the liquid crystal from which the film was drawn.

A glass frame was used and the film was drawn slowly from the neat phase with known spacings¹² in the system water, cetyl trimethylammonium bromide (CTAB) and hexanol (Fig. 1). The thickness of the film did not decrease gradually like a film from a liquid, but reached the black film state discontinuously from large thicknesses. Consequently, in the calculation of thickness the Rayleigh equation had to be applied directly after determination of the intensity of the laser beam. The results will be less accurate than those obtained using a combination of the intensity from the silvery and the black film. The com-

Structure

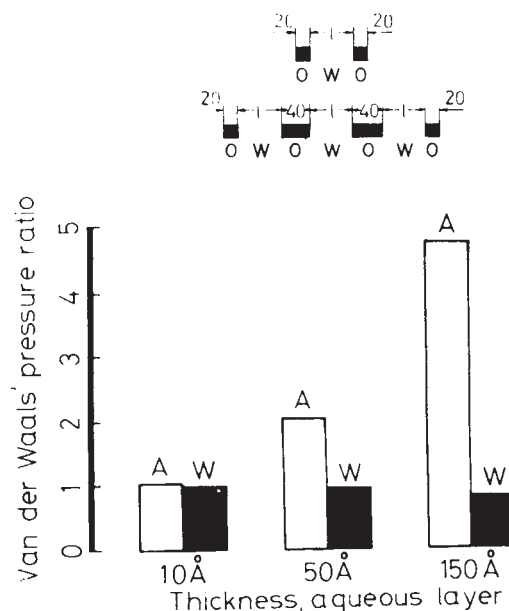


Fig. 3 The ratio between structures I and II of the Van der Waals pressure over the central aqueous layer. W (upper part of the figure), water layers, the thickness of which was varied according to the lower part of the diagram. White columns denoted by A imply infinite layers of air outside the outer oil layers, black columns represent corresponding water layers.

parison of the value from the two methods for a thin film from a solution gave, however, a difference of less than 3%. The thickness was corrected according to Frankel and Mysels¹³.

The results show that the film was thinner than the corresponding distance in the liquid crystal. This difference became more marked as the thickness of the lamellar unit increased (Fig. 2). One reason for this behaviour was found in the change in Van der Waals pressure when the adjacent layers in the liquid crystal were replaced by air to give the thin film. The pressure over the central aqueous layer was calculated using the Lifshitz approach¹⁴ with the extensions and approximations of Parsegian¹⁵. Increase of the Van der Waals pressure over the central layer occurred when three bilayers were reduced to one; pressure increased rapidly with the thickness of the layer (Fig. 3). The pressure change when the number of layers was increased from three to higher numbers was negligible.

A thin film in water gives a slight decrease of the Van der Waals pressures in the last stages of thinning (Fig. 3) and the liquid crystal in the water did not thin.

Our results show that the liquid crystal may well serve as a good model for a thin film surrounded by water; in air the usefulness seems to be limited to films which have a central aqueous layer less than 50 Å thick. The use of a lamellar liquid crystal as a model to clarify the structure of biological membranes thus seems justified.

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Excess ²⁶Mg in the Allende Meteorite

VARIATIONS in the isotopic abundance of magnesium (78.6% ²⁴Mg, 10.1% ²⁵Mg, 11.3% ²⁶Mg) have been sought^{1,2} in meteorites because of the possible addition of radiogenic ²⁶Mg by the decay of ²⁶Al. This short-lived nuclide (half-life 7.4×10^5 yr) is formed by proton bombardment of light elements³ and is of particular interest in theories of nucleosynthesis and heat generation during the early history of the Solar System. Analysed material must have a high Al/Mg ratio to avoid the dilution of any radiogenic ²⁶Mg by common Mg. Consequently, previous workers have chosen mainly feldspars from basaltic achondrites and metamorphosed chondrites. The apparent absence of excess ²⁶Mg in such meteorites is, however, not surprising, because the condensation of chondritic material from the solar nebula now seems⁴ to predate its later metamorphism by some 80 Myr, and the magmatic activity that produced the basaltic achondrites is younger still. Thus, the feldspars that formed as a result of these processes would incorporate aluminium that was separated from a nucleosynthetic event by at least 80 Myr and because of its short half-life insufficient ²⁶Al would remain to generate a detectable ²⁶Mg anomaly.

A new possibility for the generation of magnesium isotope anomalies has arisen from the discovery by Clayton *et al.*⁵ of excess ¹⁶O in the Allende Meteorite, and from

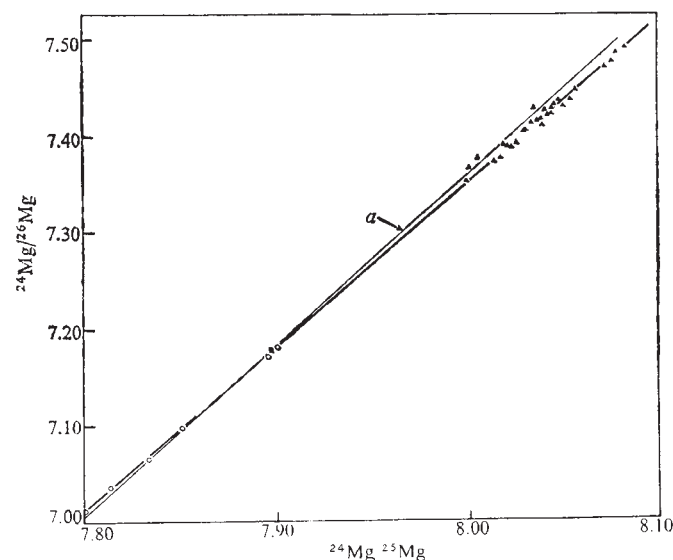


Fig. 1 ²⁴Mg/²⁶Mg against ²⁴Mg/²⁵Mg as measured by silica-gel² (open triangle) and triple-filament⁷ (open circle) runs for the same terrestrial pyroxene; the runs were chosen to show extreme isotopic fractionation. a, One stage Rayleigh fractionation; solid square, absolute value. The mass spectrometer was a single focusing, 23 cm, 60° sector machine designed for high ion transmission¹⁰, with magnetic field switching, Faraday cup collection and a calibrated digital readout for the electrometer. Peaks other than ²³Na and ²⁷Al were never observed in scans of up to 10 mass units above and below ²⁴Mg, so that spectral interference from hydrocarbons and doubly charged ions can be discounted. Data were collected when the ²⁴Mg beam current was within the range 5×10^{-12} and 5×10^{-11} A. Each point in both Figs 1 and 2 comprises the mean of a set of fifteen ²⁴Mg/²⁶Mg ratios and the combined mean of one preceding and one following set of fifteen ²⁴Mg/²⁵Mg ratios. The data are dispersed because of isotopic fractionation produced by diffusion and evaporation during the ionisation processes in the mass spectrometer. Only silica-gel runs were made with the meteoritic samples, as this mode of ionisation is far more sensitive. Sample sizes were of the order 1×10^{-6} g Mg, and laboratory contamination was approximately 7×10^{-9} g, mainly from the silica gel, which caused a maximum correction of 0.03% to two runs on chondrule 1. Magnesium was separated from the samples by standard silicate dissolution procedures followed by cation exchange in HCl.

their prediction of possible excess ^{24}Mg derived from stellar nucleosynthesis.

The Ca-Al-rich chondrules in the Allende Meteorite are the oldest objects known⁴. They are comparable chemically to the initial condensates from the solar nebula⁶, and their age, chemistry (high Al/Mg ratios) and unmetamorphosed character make them promising candidates in the search for radiogenic ^{26}Mg .

Variations in the isotopic composition of magnesium may result from mass-dependent fractionation in physical and chemical processes, and from nuclear effects. Mass-dependent fractionation is unknown in nature, but it occurs in the mass spectrometer used for isotopic ratio measurement. That, and the variation with time during mass analysis, make the determination of absolute isotopic ratios exceedingly difficult. The changes in the various magnesium isotopic ratios observed during measurement are, however, correlated with each other, because of the essential mass-dependence of the isotopic fractionation process. Measured isotopic ratios from runs on a terrestrial pyroxene illustrate the effect (Fig. 1). All points fit the same line even though two different methods of ionisation were used, and 'absolute' magnesium isotopic ratios⁷ also fit. It is curious that the slope of the line, 1.695 ± 0.013 (σ), from the pooled data of all runs including those on meteorite samples, is less than the calculated slope for the single stage Rayleigh distillation (1.79 for Mg) that is usually fitted by the isotopes of other elements. That can be attributed, however, to the operation of a multistage fractionation process. A sample with an excess of one or more magnesium isotopes because of a nuclear effect would also define a line of the same gradient, but it would be displaced from the terrestrial line. Thus, a nuclear isotopic anomaly can be detected despite the effects of variable isotopic fractionation during mass analysis. Furthermore, a numerical correction for fractionation can be made by adopting an arbitrary standard value for one of the magnesium isotopic ratios, for instance, $^{24}\text{Mg}/^{25}\text{Mg}$, and calculating the so-called 'normalised' value for $^{24}\text{Mg}/^{26}\text{Mg}$ by translation along the fractionation line. In the case of magnesium it is essential to use the empirical slope, 1.695.

We have analysed a melilite-bearing chondrule⁸ (1) comprising large melilite crystals in a matrix of very finely crystalline grossularite; an aggregate⁸ (2) containing spinel and enstatite; and an aggregate⁸ (3) containing spinel and diopside. It was not possible to remove all of the adhering matrix from the last sample, and the analysed material includes some foreign magnesium.

Minerals were identified using X-ray powder diffraction. Interpretation of the photographs was, however, difficult and unrecognised phases are doubtless present. Chemical analyses by electron microprobe are given in Table 1.

Table 1 Chemical composition of Allende inclusions

	Inclusion 1		Inclusion 2	Inclusion 3
	Melilite	Bulk*	Bulk*	Bulk*
SiO ₂	25.4	29.6	29.2	36.4
TiO ₂		1.6	0.5	1.1
Al ₂ O ₃	29.9	35.2	35.5	21.5
FeO		2.4	2.1	12.0
MgO	2.4	2.4	13.0	11.2
CaO	42.3	27.1	18.5	15.0
Na ₂ O		1.5	1.3	2.7
K ₂ O		0.1	0.1	0.1
Total	100.0	99.9	100.1	100.0
(Al/Mg) _{atomic}	9.8	11.6	2.2	1.5

* Electron microprobe analysis of a fused 2 mg sample.

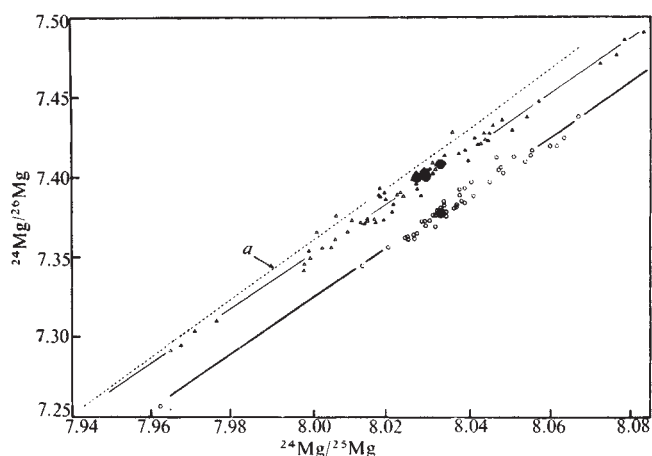


Fig. 2 $^{24}\text{Mg}/^{26}\text{Mg}$ against $^{24}\text{Mg}/^{25}\text{Mg}$ for the four runs (Table 2) of the anomalous chondrule 1 (open circles) compared with the three runs on Px 5415 (open triangles). *a*, One stage fractionation; solid circles, average for 'unfractionated' runs. The points for chondrule 1 define a separate fractionation line lower than the pyroxene line in $^{24}\text{Mg}/^{26}\text{Mg}$ but with the same mean $^{24}\text{Mg}/^{25}\text{Mg}$. All other data in Table 2 fit the pyroxene line.

All but one of the analysed samples has a terrestrial isotopic composition. The anomaly in chondrule 1 (Table 1) can be displayed on a plot of measured isotopic ratios (Fig. 2) which compares data for the chondrule with those from a terrestrial sample. The data for the chondrule fit a fractionation line that has a slope identical to the terrestrial line (respectively, 1.70 ± 0.04 and 1.68 ± 0.02) but is displaced from it towards lower $^{24}\text{Mg}/^{26}\text{Mg}$. Thus we have detected a nuclear effect in the magnesium isotopic composition of chondrule 1.

Five out of fifteen runs showed no variable isotopic fractionation. They are termed 'unfractionated' in Fig. 2 although they are clearly biased with respect to the Catanzaro⁷ 'absolute' values. For these runs, as well as for the means of the fractionated runs, the ratios $^{24}\text{Mg}/^{25}\text{Mg}$ are all nearly the same. This fact strongly implies that the principal component of the nuclear effect in Fig. 2 is undoubtedly an excess of ^{26}Mg . The entire effect may result from the addition of a pure ^{26}Mg component to common magnesium. Our data do not, however, exclude the possibility that very small amounts of ^{25}Mg and/or ^{24}Mg may be present in the added ^{26}Mg -rich component as well. The $^{24}\text{Mg}/^{25}\text{Mg}$ ratio of this hypothetical magnesium could be greater or less than in terrestrial magnesium.

Because any difference between the $^{24}\text{Mg}/^{25}\text{Mg}$ ratios of chondrule 1 and common magnesium is so small, the results can be described by normalising values of the $^{24}\text{Mg}/^{26}\text{Mg}$ ratio to a fixed $^{24}\text{Mg}/^{25}\text{Mg}$ value. For that we have chosen our mean measured $^{24}\text{Mg}/^{25}\text{Mg}$ (8.03) rather than the 'absolute' value thus minimising the shifts which result from normalisation and also any error resulting from uncertainty in the slope of the fractionation line (Table 2). The terrestrial isotopic composition was determined in two pyroxenes, six runs giving a mean $^{24}\text{Mg}/^{26}\text{Mg}$ ratio of 7.404 ± 1 . The most precise of the runs seems to be an outlier (Px 5414 R2) but there are no grounds for its rejection. Measurements on the total Allende Meteorite fall into the range of the terrestrial population (mean 7.406 ± 1) as does that for aggregate 2 (7.406 ± 2). The mean ratio for aggregate 3 (7.402 ± 1) is slightly lower than the terrestrial value and may be anomalous, although it is matched by the outlying terrestrial measurement. Two separate dissolutions of chondrule 1 were both analysed twice and the results agreed excellently. The mean value of (7.374 ± 1) is quite distinct from the terrestrial range, indicating a 0.41% anomaly.

There are very obvious needs to find anomalous magnesium in other Allende inclusions; to find the particular mineral phase(s) which bears the anomaly; and to relate the magnesium anomaly to the excess ^{16}O found by Clayton *et al.*⁵ Nevertheless, some conclusions can still be drawn. First, because of the constant $^{24}\text{Mg}/^{26}\text{Mg}$ ratio, it is likely that the magnesium anomaly of chondrule 1 results entirely from excess ^{26}Mg , and this in turn implies strongly an origin by the radioactive decay of ^{26}Al formed during intense proton irradiation in the early stages of the Solar System. As the ^{26}Al must have been made within the Solar System because of its short half-life, then it is likely that the excess ^{16}O in the Allende Meteorite and also other short-lived nuclides such as ^{129}I (ref. 9) were formed within the Solar System, rather than outside it⁵. Second, the absence of a comparable magnesium anomaly in the other two chondrules can be attributed easily to their low Al/Mg

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Table 2 Normalised $^{24}\text{Mg}/^{26}\text{Mg}$ ratios*

Sample	Dissolution	Run	Number of ratios	Mean $^{24}\text{Mg}/^{26}\text{Mg}$
Terrestrial pyroxene				
Px 5415	Mg4	R2	300	$7.400 \pm 1 \uparrow$
		R4	180	7.404 ± 3
		R5	480	7.404 ± 2
Px 5641	Mg3	R1 $\dagger$	195	7.405 ± 2
		R2	240	7.404 ± 2
		R3	225	7.407 ± 2
Allende Meteorite				
Total meteorite	Mg1	R2 $\dagger$	240	7.406 ± 2
		R3 $\dagger$	225	7.407 ± 3
Inclusion 1	Mg1	R1	180	7.375 ± 3
		R2	105	7.372 ± 3
	Mg2	R1	345	7.374 ± 1
		R3 $\dagger$	270	7.374 ± 2
Inclusion 2	Mg1	R1	135	7.406 ± 2
Inclusion 3	Mg1	R1	345	7.401 ± 1
	Mg2	R1 $\dagger$	330	7.402 ± 1

* $^{24}\text{Mg}/^{26}\text{Mg} = 8.03$.

† Uncertainty is the 95% confidence limits for the mean and refers to the last digit.

‡ Runs with no variable isotopic fractionation.

ratio (Table 1). This strengthens the idea that the anomaly results entirely from radiogenic ^{26}Mg . On the basis of a 0.4% excess of ^{26}Mg for chondrule 1, excesses may be expected of 0.08% and 0.05% for aggregates 2 and 3, respectively, from their Al/Mg ratio, of which the 0.8% excess may just be detectable (compared with 0.035% for the coefficient of variation of the six pyroxene runs). If the failure to detect an anomaly in aggregate 2 is not because of insufficient data, two other possibilities may be suggested: the introduction of common magnesium after the assembly of the Allende Meteorite (alkalis have been added to many such chondrules, particularly to aggregates, after more than 10^9 yr⁴); or the condensation of much of the material of inclusion 2 at a later time than that of chondrule 1. Finally, we note the value 3.8×10^{-3} that may be calculated for the ^{26}Al ratio in chondrule 1 at its time of chemical isolation. That may be related to the radioactive heating of planetesimals² and provided that accretion occurred immediately after the formation of ^{26}Al , it is more than sufficient to account for the heating to metamorphic temperatures of chondritic planetesimals.

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Magnetic telechemistry is elegant but nature is complex

Vogt and Johnson¹ have suggested that the bulk chemistry of *in situ* abyssal basalts may be diagnosed using sealevel magnetic anomalies. Their suggestion is based on two sets of assumptions and observations.

First, they assume that the concept of mantle 'plumes'^{2,3} is a valid way of explaining the existence of selected oceanic island platforms and other positive elements on the sea floor, and that 'plumes' are associated with increased magnetic anomaly amplitudes.

The second assumption is that the observed increases of magnetic anomalies which are associated with the Galapagos positive element, or 'plume', result from a relative increase in Fe and Ti concentrations. The assumption is based on a comparison between published chemical data and anomaly amplitudes for the Juan de Fuca and Gorda ridges.

In contrast to Vogt and Johnson's assertion, however, oceanic platforms (the most idealised surface expression of 'mantle plumes'), or 'hot spots'^{4,5}, do not generally have magnetic anomalies of significantly higher amplitude. A characteristic of most island platforms is the general loss of the high amplitude linear magnetic anomalies which are normally caused by dyke injection during seafloor spreading^{6,7}. This is probably because the extrusive igneous activity increases relative to intrusive activity during the formation of the island platform; the process also involves hydro-explosions⁸ as the platform becomes shallower than 500 m (ref. 9). A probable concurrent loss of a regular stress field also occurs, because of the mass of the platform on the seafloor¹⁰. Vogt and Johnson recognise that problem: they comment that magnetic anomalies above the Cocos Ridge and Carnegie Ridge, associated with the Galapagos 'Plume' are low amplitude. So they invoke the local existence of non-magnetic layers of tephra, and layers with opposing (cancelling?) polarities. Similarly, Campsie *et al.*³ have suggested that these ridges may be chemically composite: partly 'plume' and partly normal oceanic ridge materials. These special arguments detract

severely from Vogt and Johnson's idea. Their high anomaly-mantle plume association is based on high and low magnetic anomalies observed over the Juan de Fuca and Gorda ridges, respectively, which they claim are 'plume' and 'non-plume' material, respectively. An equally feasible explanation is that the standard deviation of the width of the zones of active dyke injection^{6,7,11} is narrower for the Juan de Fuca Ridge than it is for the Gorda Ridge.

Although there is a direct relationship between the FeO content (%) of dredged samples and the amplitudes of the magnetic anomalies for the Juan de Fuca Ridges (ref. 1: Fig. 2), there is no increase in the amplitudes of the anomalies over the Iceland 'plume' despite the high Fe and Ti content in the dredged rocks. The absence of expected correlation is attributed to an 'anomalous' situation: "increasing ash content and lava fracturing at low hydrostatic pressures". This does not, however, prevent Vogt and Johnson from using chemical data from the Rekjanes Ridge¹² in order to extend their idea to a possible inference of several different elemental concentrations.

Although Vogt and Johnson clearly state their assumptions, much evidence exists to show that a constant Fe/Ti ratio could not be maintained reasonably during an unequal increase in the total concentrations of FeO and TiO₂ in basalt. Variation of the intensity of magnetisation, J , within the FeO-Fe₂O₃-TiO₂ system¹³, shows that factors such as oxygen fugacity, degree of quenching, and oxyexsolution, are much more important controls of J than simple FeO and TiO₂ concentrations^{14,15}, unless order of magnitude differences are involved. Of major importance is the fact that the Curie point decreases to below room temperature with increasing TiO₂ content and decreasing oxidation state of Fe, thus rendering as minimal the prospects of high remanent J in such rocks. Magnetic stability (which depends primarily on magnetic grain size^{14,16}) is also important: if it is absent, then no reversed polarity would persist and the essential polarity variation required for the production of high amplitude anomalies would not occur. Unless of single domain size, Fe is quite unstable, and so an increase of Fe alone would not necessarily contribute to an increase in anomaly amplitude. For Johnson and Vogt's proposal to be correct, it would (as they state) be necessary for 'plumes' to generate greater amounts of titanomagnetite, with relatively unvarying Fe/Ti ratios, and grain sizes. But these parameters are to a large extent a function of the cooling rates of the igneous bodies involved, so it seems very unlikely that they would remain constant between those bodies which constitute abyssal crust and oceanic platforms, which have inherently different modes of emplacement. Different modes of igneous emplacement alone can vary drastically the amplitude of magnetic anomalies¹⁰, and as primary chemical properties do not depend on the mode of emplacement, there is no reason whatsoever to believe that they are the controlling factors in the character of all magnetic anomalies.

Despite the elegance of Vogt and Johnson's idea, I suggest that insufficient rigour had been applied to the concept of magnetic telechemistry.

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DRS VOGT AND JOHNSON REPLY: We re-emphasise that we never attributed all of, or even most of, the amplitude variations of oceanic magnetic anomalies to the chemistry of basalt. We simply proposed that chemistry does play a role in certain areas and should not in any case be ignored as a possible factor. Even if the hypothesis explains only three areas of high amplitude anomalies¹ this amounts to about 8×10^5 km² of oceanic crust, certainly more than a very restricted marine geological circumstance.

At the outset Watkins wrongly asserts that 'magnetic telechemistry' is based on the acceptance of 'mantle plumes'. But the connection between high magnetic anomalies and high Fe-Ti concentration never depends on whether the geochemical anomaly is the result of differentiation in the uppermost mantle² or on whether there are two distinctive 'primary' (Fe-Ti rich) and 'depleted' magmas in the mantle³. The hypothesis does not depend on the fixed position of the hot spots with respect to the Earth's spin, nor on whether there is deep rising convective motion below the hot spots⁴. But areas of high magnetic anomaly are found in association with some hot spots, and so the two phenomena are apparently related.

Hot spots can be 'Icelandic' (close to spreading axes) or 'Hawaiian'⁵; only Icelandic hot spots can have a 'telechemical' signature. The topographically positive element of Icelandic hot spots really consists of at least two parts. First, there is an aseismic ridge or platform, or volcano chain, distinctly, and relatively sharply, elevated above the surrounding oceanic crust. Examples are the Cocos, Carnegie, and Iceland-Faeroe ridges and the seamount chains extending north-westwards from the Juan de Fuca Ridge. These ridges represent, in the stationary hot spot hypothesis, trajectories of plates over the deeper mantle. Such ridges are constructed at least in part by 'Hawaiian' igneous activity: extrusive and intrusive activity away from the spreading axis. If some sort of spreading is involved in their creation, it is of a more complicated variety. Surely this is so on Iceland. These ridges usually involve an oceanic crust thicker than normal. Perhaps as a result of these differences, many aseismic ridges have confused magnetic signatures of relatively lower magnetic amplitude. There are obviously factors other than basalt composition involved, and there was never any reason to expect that an unqualified telechemical hypothesis could explain the magnetic signatures of the Cocos, Carnegie, Iceland-Faeroe, and similar ridges or platforms. These aseismic ridges are simply another and more complicated form of oceanic crust, but are distinct as far as magnetic anomalies are concerned.

The second part of the topographic anomaly is a broad, smooth, regional elevation in the spreading axis and older isochrons. It is here that we observe, at least in some cases, regions of unusually high magnetic anomalies of seafloor spreading origin. Parts of these regional bulges are also characterised by relatively smooth basement and reduced seismicity, but whereas the aseismic ridge *sensu strictu* is sharply demarcated, this broader bulge is not. In the Iceland area the

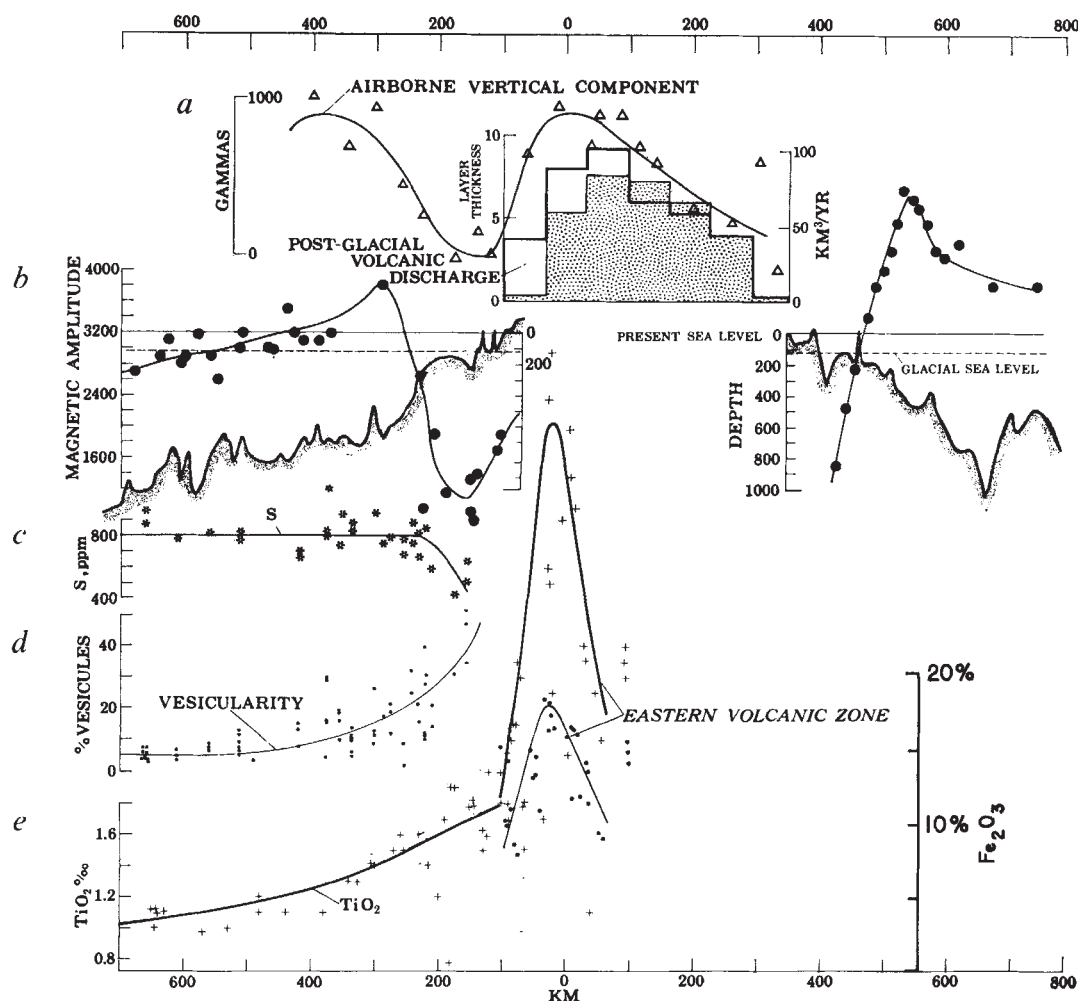


Fig. 1 Chemical and physical parameters plotted against distance from the centre of the Iceland plume along the axis of spreading. North-east to the right. *a*: Triangles, maximum vertical component¹⁰ magnetic anomaly relief (axial high to negative west of it along Reykjanes Ridge); heavy line histogram, postglacial volcanic discharge ($\text{km}^3 \text{yr}^{-1}$), (ref. 8). *b*, Axial magnetic high of the western negative (from the shipborne profiles^{11,15}); *c*, sulphur content⁶; *d*, vesicularity⁸; *e*: Fe_2O_3 (filled circles), and TiO_2 (crosses) along the Reykjanes Ridge³ and eastern neovolcanic zone of Iceland⁸. Bathymetric profile along the axis was constructed from United States Naval Oceanographic Office charts. Note the approximate correspondence in peaks of TiO_2 , Fe_2O_3 , discharge, and the maximum vertical component of the magnetic anomaly. Where these quantities peak may mark the plume centre. There is a sharp drop of anomaly amplitude as the spreading axis rises to within less than about 400 m of sea level. This may reflect increasing oxidation states of titanomagnetites as volatiles are lost at low pressures¹⁶, as evidenced by the high vesicularity⁷ and low sulphur⁷ in the same region. Between 300 and 700 km south-west and 550 to 750 north-east the systematic variation in the anomalies is primarily the result of varying source depth.

regional bulge extends southward through the Reykjanes Ridge to the Gibbs Fracture Zone, some 2,000 km from the centre of Iceland; it seems to extend a similar distance northwards. The Greenland-Iceland-Faeroe Ridge, although concentric with the broader bulge, is an order of magnitude narrower. Similarly, the Cocos and Carnegie ridges are only 50–100 km wide whereas the topographic bulge of the Galapagos Ridge, centred north of the Galapagos Islands, extends 500–600 km westwards, and eastwards and is up to 500 km wide at the longitude of the islands. The Juan de Fuca Ridge is 500 km long, much more extensive than the width of seamount chain(s) terminating near its axis.

The Iceland area (Fig. 1) is, Watkins claims, another 'exception' to our hypothesis. In fact, the Mid-Oceanic Ridge rises above sea level there, and there was no *a priori* reason to expect magnetic anomalies not to be affected. It has been known for a long time that the magnetisation of subaerial and suboceanic basalts is quite different, even if initial magma compositions

were identical. Beginning at depths of 500 m or so, and increasing at shallower depths, vesicularity and explosiveness of the lavas increase as they erupt under lower confining pressures. The greater fracturing and ash content would decrease the bulk magnetisation of the oceanic crust¹; degassing at vesicularities above 15% (~500 m depths) results in reduced sulphur concentrations⁶. High *J* values of oceanic pillow basalt are possible because rapid quenching allows the retention of volatiles and the maintenance of titanomagnetites in low oxidation states⁷. As the Reykjanes Ridge shoals toward sea level, volatiles are lost (Fig. 1) and there is every reason to expect magnetic amplitudes to decrease. The observed dramatic decrease in amplitude of the axial anomaly as the spreading axis shoals toward Iceland (Fig. 1) means, if our telechemistry argument¹ is valid⁴, that the degassing effect overpowers the opposing effect expected from the increasing^{3,8} Fe and Ti content. Fluctuations in Pleistocene sea level would have smeared out the decline in magnetic amplitudes beyond that

expected from sampling only post-glacial basalts. Glacial erosion could also have removed some of the magnetised layer.

Unfortunately, the Ti and Fe concentrations decrease linearly south-westwards, away from Iceland so steeply that there is only a small section of the Reykjanes Ridge deeper than the degassing depth (as defined by reduced S concentrations) but still anomalously high in Ti and Fe. In that small section Ti and Fe are only slightly above the normal levels occurring further south on the Reykjanes Ridge, and any increase in anomaly amplitude in that small section may be within the noise level. The observed increase of amplitude as Iceland is approached from either side seems to be accounted for largely by the changing source depth. We are investigating this.

As degassing should not, in general, affect concentrations of rare earths, trace and minor elements such as Fe and Ti, and as the Reykjanes Ridge is the only geochemically anomalous region for which detailed data exist, we adduced the Reykjanes chemical data³ in support of our hypothesis, in spite of the absence of high magnetic amplitudes. This absence does not demand a new *ad hoc* hypothesis on our part nor is it an additional latitude as construed by Watkins⁹. In fact, low pressure degassing is a second kind of 'magnetic telechemistry', which reduces *J* and therefore magnetic amplitudes. It is uncommon because the mid-oceanic spreading axis rarely approaches within 500 m of sea level.

Watkins implies that the differences on magnetic amplitude observed in the Galapagos, Juan de Fuca and Pacific-Antarctic areas¹ are perhaps explained more plausibly by differences in thickness of the magnetised layer, mode of emplacement, oxygen fugacity, degree of quenching, and oxyexsolution. The thickness of the magnetised layer is not measured readily as its meaning in terms of seismically determined crustal structure is still somewhat obscure¹¹. In any case, there is no evidence that the crust in the anomalous areas discussed is thicker than elsewhere. We are talking about regional bulges of oceanic crust produced by simple spreading, not about aseismic ridges with thickened crusts. If a thicker layer provides the explanation, deep tow magnetic/seismic profiling would reveal the same apparent magnetisation of minor basement features in both areas. But profiles collected in the Galapagos area show that the high-amplitude region has more intensely magnetised basement irregularities¹¹, and Fe/Ti basalt also has higher *J* values in the few dredge samples available¹¹. Basement depth is generally continuous across the regional boundary, and both kinds of lavas are exposed to the same physical environment when they erupt. There seems no reason to postulate that the other factors cited by Watkins are necessarily important variables but it is also hard to show that they are not. The advantage our telechemical argument has is that chemical differences have been reported from all hot spots so far studied.

Differences in the mode of emplacement cannot be discounted entirely, especially in the case of the Juan de Fuca Gorda ridges with their different morphologies and different levels of seismicity¹². If the zone of active igneous activity is broader, anomaly amplitudes fall, and the resolution of short-period reversal events deteriorates^{13,14}. It is doubtful, however, whether this mechanism could explain the uniform amplitude difference between the Juan de Fuca and Gorda ridges. In the Galapagos area, no such deterioration is evident and the width of the 'variable dyke zone' hypothesis seems quite unlikely.

So until a more plausible hypothesis is advanced, we stick with magnetic telechemistry. This does not mean that the two regions (of high and average amplitude respectively) are likely to differ only in their bulk chemistry. Nor does it mean that magnetic amplitudes are necessarily a linear function of Ti and/or Fe. For example, if the rate of increase of magnetisation decreases with increasing iron content, then relatively uniform, high amplitude provinces, within which there is substantial geochemical variation, would result. Should the analysis of rock samples demand such a complex relationship

between chemistry and magnetic anomalies—possibly involving additional 'hidden' variables—it would in no way demolish our hypothesis.

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The reaction of monkeys to 'fearsome' pictures

THE phenomena a man or animal most needs to know and understand must often be potentially dangerous or discomfiting. We report here evidence that monkeys, given the opportunity to look at a picture which excites both interest and fear, choose first to look at it and only later, once their interest has abated, to avoid it.

We have measured the preference for a visual stimulus by allowing the monkeys to choose between looking at the test stimulus and a blank white screen of the same subjective brightness. In earlier experiments^{1,2} we found that the response to an informative but affectively neutral stimulus, for example a repetitive loop of cartoon film, was typically an initial strong positive preference which declined within a few hundred seconds to relative indifference. By contrast, the response to an uninformative but unpleasant stimulus, such as a plain field of red light, was a strong and stable aversion. When a stimulus was both informative and unpleasant, for example a black and white film loop projected through a red filter, the monkeys' 'interest' overrode—so long as it lasted—their 'unpleasure'. The purpose of the present study was to find out what would happen with stimuli which now were deliberately chosen to be both informative and 'scaring'.

A television screen was used for displaying the stimuli, which were recorded on video-tape loops so that each action sequence was repeated approximately every 10 s. We made up 15 potentially fear-evoking stimuli, selected partly on the basis of our own hunches and partly on the basis of Hebb's suggestion³ that 'anomalous' objects give rise to fear in primates. The stimuli included a toy snake, burning paper, a lavatory brush, a mop-head wearing a human mask and

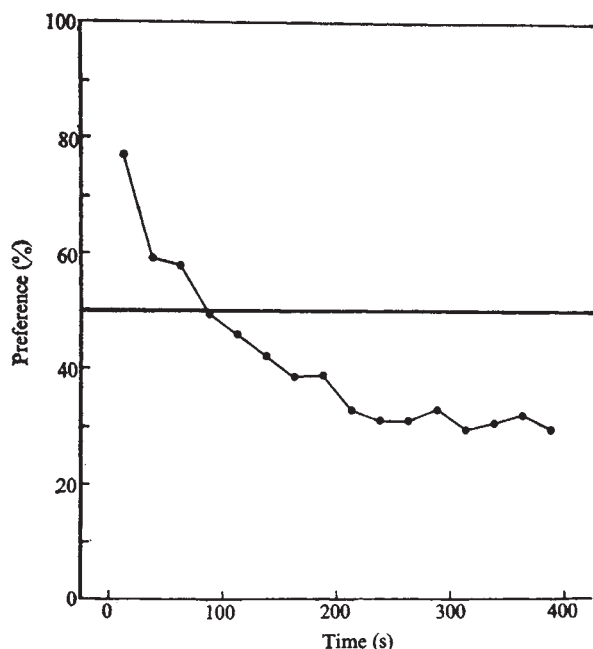


Fig. 1 Preference for 'fearsome' pictures (preference measured as the ratio of the total time spent with the test stimulus to the total time spent with either the test stimulus or the blank white screen).

other objects similarly bizarre. Each object was given some kind of life-like motion.

The method for measuring the monkeys' preferences has been described in detail in earlier papers^{1,2}. The monkey sat in a small dark chamber with a television screen (37 cm × 30 cm) at one end, taking up most of the wall. On the screen could be displayed either the test stimulus or a blank white field of the same subjective brightness. The monkey controlled the presentation of the two alternative stimuli by pressing a button: successive presses on the button produced the two stimuli in strict alternation, the stimulus staying on as long as the monkey held the button down. The test was terminated after 400 s exposure (at which point two peanuts were delivered). The monkeys worked eagerly and generally completed the test in under 500 s, alternating rapidly between the two stimuli (on average about 30 alternations per 100 s of exposure).

The subjects were five young male rhesus monkeys (*Macaca mulatta*). All had taken part in previous experiments on preference, in the course of which they had completed several hundred tests and had become thoroughly familiar with the apparatus and with the fact that strange stimuli were likely to be presented to them. They had, however, never before been deliberately exposed to fear-evoking stimuli.

The monkeys were tested with two new stimuli each day over a period of three weeks. It was immediately apparent from the results that it was not so easy to scare the monkeys as we had imagined. In fact in many of the tests there was no sign that the monkeys were anything other than interested in the stimuli. Since our aim was specifically to study the interaction of interest and fear, we needed to select for analysis only those tests in which we could be reasonably sure that the monkeys were indeed afraid. Independent indices of distress (cries, urination and so on) showed that the stimuli which the monkeys found most upsetting were, as might be expected, those to which they showed the greatest overall aversion. We decided therefore to treat any test in which the monkey's preference was predominantly negative as a case of the monkey being scared, and accordingly we defined a stimulus as being 'fearsome' to a particular individual if he chose to look at it for less than 200 s of the 400 s test. By this criterion, 13 of the 15

stimuli turned out to be fearsome to one or more of the monkeys, while the five individual monkeys were scared of eight, seven, seven, three and three of the stimuli, respectively.

Figure 1 shows the typical pattern of preference for 'fearsome' stimuli, as defined above, in each successive 25 s of the test. To obtain this graph we took for each monkey, the mean of his preference for those stimuli which were fearsome to him and then the mean of these means for the five animals. The point to notice is the time course. The characteristic pattern was for aversion to appear only after a short-lived positive preference. This pattern was fully borne out by the data from the individual tests: of the 28 tests in which the stimulus proved fearsome, the preference, taken over each successive 50 s, was positive for the first 50 s in 24 instances and negative for the last 200 s in every instance.

The pattern of preference was very similar, at least in quality, to that obtained with the red film loops in the earlier study². We showed previously that the response to 'red films' could be accurately accounted for by a mathematical model which treats 'interest' and 'pleasure/unpleasure' as two separate factors which interact to determine behavioural preference, subject to a combinatorial rule which gives precedence to 'interest'. Our results suggested that the response to fearsome pictures might likewise be dually determined, and the intriguing possibility arose that we might be able to 'synthesise' the curve of Fig. 1 by using suitably chosen 'compound' stimuli in which we could separately identify components of interest and unpleasure.

To this end we undertook a second experiment in which we presented the monkeys with non-fearsome television pictures to which an unpleasant auditory stimulus ('white noise') was added. The non-fearsome pictures consisted of 10 s video loops of relatively bland material, chosen to be roughly comparable in information content to the fearsome

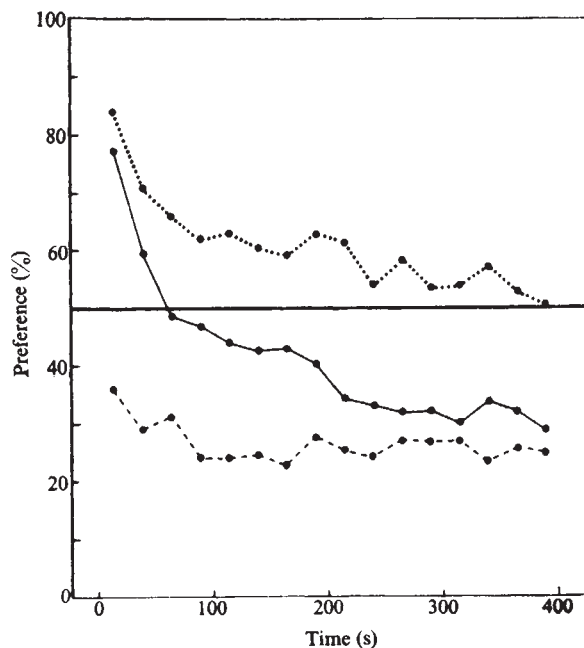


Fig. 2 Preference for, ●—●, 'picture only' (a choice between a non-fearsome, silent, video loop and the blank white screen); ●---●, for 'noise only' (a choice between the blank white screen plus white noise and the blank white screen without the noise); ●·····●, for 'picture + noise' (a choice between a non-fearsome video loop plus white noise and the blank white screen without the noise). Monkeys were given 10 tests in each category, taken in rotation. Graphs show the means of the mean preferences of the five animals.

pictures. The white noise was set at an intensity level of 68 db (16 db above the background level of the testing chamber) which preliminary observations indicated would create an appropriate degree of displeasure.

The monkeys were given three categories of tests: 'picture only', 'noise only' and 'picture plus noise'. As Fig. 2 shows, the response to the pictures only was an initial strong positive preference which declined towards indifference; that to the noise only was a steady aversion (increasing slightly over the first 100 s of the test); that to the pictures plus noise was an initial positive preference which then turned into a marked aversion. In line with the predictions of the mathematical model, the response to pictures plus noise could be almost perfectly fitted by a theoretical curve computed from the separate responses to pictures only and noise only¹.

Comparison of Figs 1 and 2 illustrates the close correspondence between the response to the fearsome pictures and that to the non-fearsome pictures plus noise. It seems fair to say that the 'fearsomeness' of the fearsome pictures had on average the same effect as 68 db of white noise. But we believe there are grounds here for a stronger assertion, namely that at a causal level fearsomeness influences behavioural preference in the same way as noisiness (or redness) through the evocation of a common factor of 'unpleasure', a factor which is strictly subservient to 'interest'.

In functional terms the lesson of these results seems clear: the benefits that come from increased understanding outweigh the immediate rewards of a comfortable life.

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Behavioural thermoregulation in a wolf spider

LABORATORY studies have shown that wolf spiders (Lycosidae) may prefer temperatures above the ambient air temperature of their normal environment^{1,2}. They are commonly active on clear days in winter and may repeatedly move in and out of the sun. In addition, they carry their egg sacs on their spinnerettes and apparently incubate them in the sun^{3,4}. While the observations suggest that these spiders may exhibit behavioural thermoregulation, it has not been demonstrated.

During a study into the ecological energetics of the large, burrow-inhabiting wolf spider *Geolycosa godeffroyi* (L. Koch 1865) in the Australian Capital Territory⁵, continuous temperature records were obtained of both the egg sac temperature and the temperature of the cephalothorax of the spiders.

Egg sac temperature was measured by implanting a temperature transmitter into the egg sac after the removal of about 70% of the eggs. The egg sac was returned to the female in the field and the transmissions monitored during the daylight hours. The resulting magnetic tapes were transcribed at intervals and records obtained from six individuals. The temperature of the spiders was recorded in the field by implanting a fine thermocouple junction into the cephalothorax and recording the temperature with an electric thermometer with an external reference junction

at 0° C. The fine thermocouple lead (2.3 mg cm⁻¹) allowed the spider free movement within the burrow and through its normal activity range. The ambient air temperature was recorded from the dry bulb of an aspirated hygrometer placed 1 cm above the ground surface. Temperatures were recorded for 24 h periods from spiders weighing 0.1-1.5 g from February to November 1972.

Figure 1 shows two representative records of the cocoon temperature in summer. Figure 1b is the record for an intermittently overcast day showing that the egg sac could achieve a considerable temperature excess⁶. In Fig. 1a the spider provided its own control. At A the spider was placed with its 'bugged' cocoon next to its burrow which was partly occluded by debris. The spider began to clear the debris from the burrow but at B dropped the egg sac in full sunlight outside the burrow and continued with its work. The sun was masked by cloud at C and when the spider had cleared the burrow it recovered the egg sac (D) and entered the burrow. Between D and E the sky cleared and the spider began to sun the egg sac at E. The burrow was lightly shaded by trees after 1500h but was bathed in full sunlight at 1800h, just before sunset. The spider maintained a fairly constant egg sac temperature in full sunlight (1200h to 1500h) but the temperature excess potentially reached was considerably greater (A to E in Fig. 1a) than that maintained by the behaviour of the spider.

Figure 2 shows three representative records of the cephalothorax temperature of the spiders at different times of the year. Figure 2b represents part of the record obtained on a subfemale on March 13, 1972. At 0600h the spider was still benefiting from the thermal lag in the soil temperature profile and by remaining at the bottom of the burrow maintained its temperature 8° C above the ambient air temperature. As the ambient temperature rose in the morning the spider came to the top of the burrow and maintained a temperature close to the ambient temperature until the sun struck the burrow entrance at about 1000h. The body temperature rose rapidly to about 40° C when

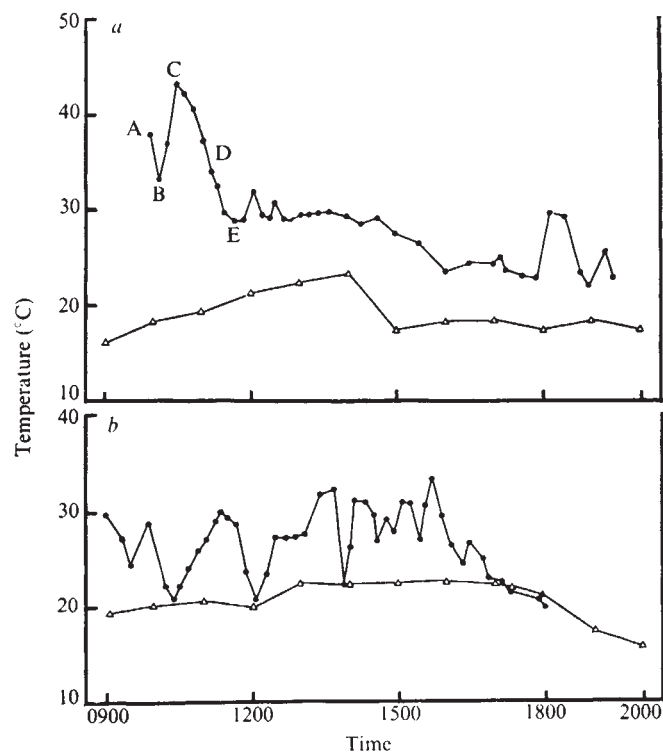


Fig. 1 Egg sac temperatures of *G. godeffroyi* obtained from implanted temperature transmitters (●) and ambient air temperatures (△). The letters A-E are explained in the text.

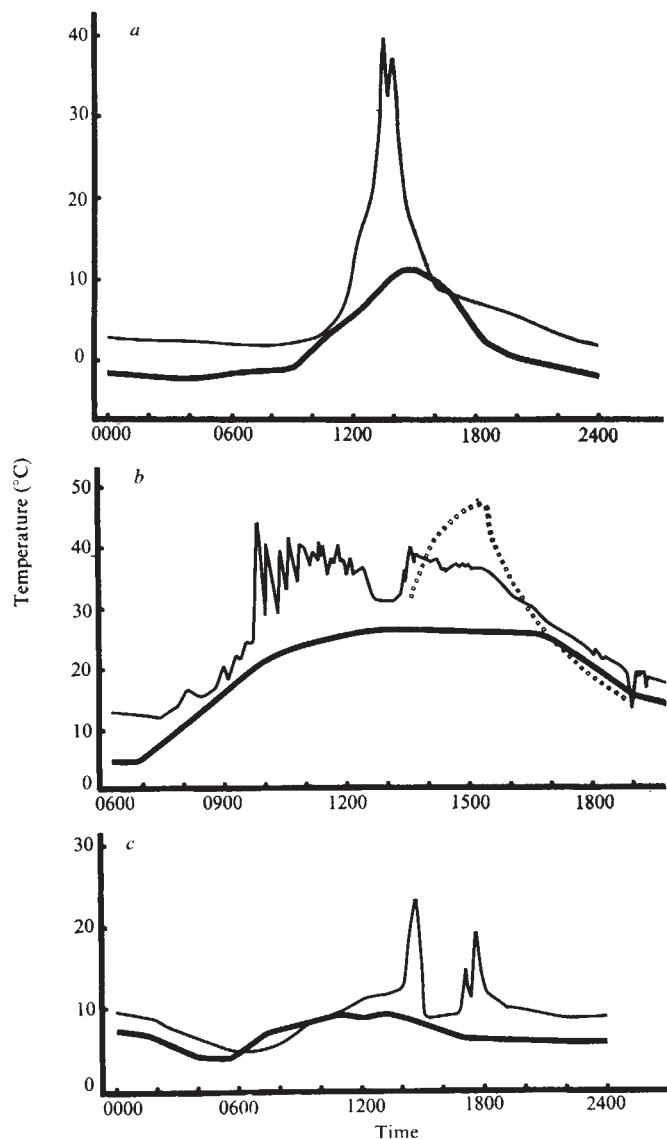


Fig. 2 Cephalothorax temperatures of *G. godeffroyi* obtained from implanted thermocouples (—) compared with ambient air temperatures (---) and dead spider temperatures (···). The records were obtained in, a, mid-winter (June 25, 1972), b, early autumn (March 13, 1972) and, c, late winter (September 13, 1972).

the spider retreated into the shade of the burrow. This process continued until about 1200h when the sun was partially obscured by cloud until 1330h. The ambient temperature dropped after 1600h and the spider retired down the burrow, save for some minor excursions up the burrow at about 1900h, and again maintained its temperature above the ambient air temperature. At 1400h a freshly killed spider of similar size was placed on the grass next to the burrow and its temperature monitored. The temperature excess obtained by the dead spider greatly exceeded that of the live spider and shows the controlling effect of the spider's movement within the burrow on the body temperature and showing, at night, the moderating influence of the burrow. The finer control over the body temperature in the afternoon was characteristic and may result from the spider adopting thigmothermic as well as heliothermic regulation. This is supported by the more gradual drop in temperature in the afternoon after the ground had been thoroughly heated compared with the rapid temperature changes in the morning and in winter (Fig. 2a and 2c) whenever the sun was obscured.

Figure 2a represents the temperature of a spider on a clear winter's day. While the ambient air temperature

reached 11°C, the spider's temperature rose rapidly to 39.7°C when the sun was on the burrow, resulting in a temperature differential of 30°C at 1345h. At night the spider's temperature fell to only 1.8°C in spite of the ambient temperature falling to -2.3°C. Figure 2c is the record from a spider on a generally overcast day in early spring and shows the immediate use the spider made of radiant energy when the sun struck the burrow at 1300h and 1800h.

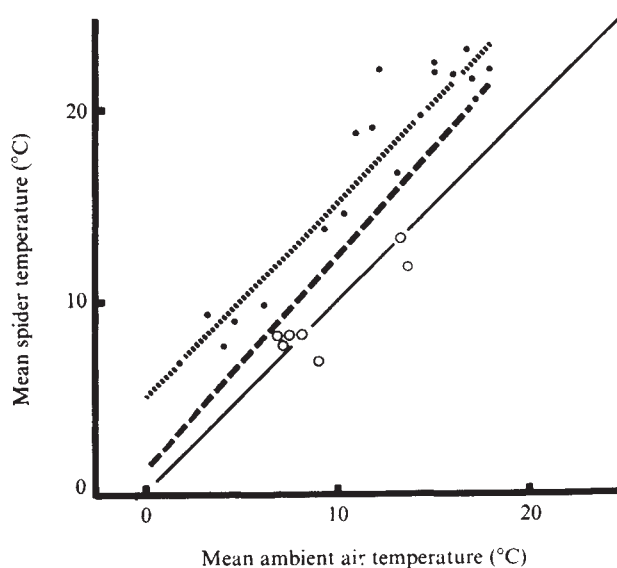


Fig. 3 The relationship between the mean daily cephalothorax temperature and the mean daily air temperature for each of 26 individual *G. godeffroyi* recorded throughout 1972. The null line (—) assumes that the ambient and spider temperatures are equal. The records are divided into those obtained on clear days (●) and overcast days (○) and the regressions shown for all days (---) and clear days (·····).

In Fig. 3 the mean daily spider and ambient temperatures are plotted for the 26 d of recording throughout 1972. The slope of regression through the data for the clear days only is not different from a slope of 1.0 ($t_{19}=0.845$, $0.5 > P > 0.4$). On clear days throughout the year the spiders maintained their body temperature an average of 4.6°C above the ambient air temperature by basking in the sun during the day and retreating down their burrows at night.

The similarity between the thermoregulation in *G. godeffroyi* and that found in some lizards⁶⁻⁸ is striking, the main difference lying in the finesse of the control in the plateau temperature. While the lizards exhibited fine control at the upper temperature, that of the spiders was erratic, probably due to their low thermal capacity.

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Functional reinnervation in kitten visual cortex

MOST cells in the adult cat visual cortex, and even in the very young kitten can be activated by visual stimuli presented to either eye¹⁻³. The retention and refinement of these normal binocular connections depends critically on simultaneous correlated visual experience through both eyes early in life. If a kitten is deprived of vision in one eye during a 'sensitive period', which stretches from about 3 weeks to 3 months, the normal pattern of functional connections is grossly disrupted; the deprived eye loses its influence and most cells can only be driven by stimuli through the experienced eye⁴. Blakemore and Van Sluyters⁵ have recently shown that this situation can be partly or totally reversed by covering the previously experienced eye and opening the deprived eye, and allowing that eye a substantial period of visual experience. Thus one set of afferent terminals, robbed of almost all influence in the visual cortex, can re-establish working connections. We have now analysed the form and rate of this functional reinnervation.

In six kittens the lids of the right eye were sutured together at or before the time of natural eye opening (normally 6-10 d). Reverse suturing was performed at the age of 5 weeks: the lids of the right eye were opened and those of the left eye sutured shut. All precautions were taken to ensure that the animals never received simultaneous binocular vision. As a control, one kitten was monocularly deprived in the right eye for 5 weeks, and recordings taken immediately.

Animals were prepared for electrophysiology under barbiturate (Brietal) or steroid (Althesin) anaesthesia. They were then paralysed with Flaxedil and artificially respiration with 75-80% nitrous oxide⁶. The corneas were covered with contact lenses and 3 mm artificial pupils, and refractive errors corrected with supplementary lenses. Single units in the visual cortex were isolated with tungsten-in-glass microelectrodes⁷, and their action potentials were conventionally amplified and displayed.

All penetrations were later shown histologically to be largely or wholly within area 17. The electrode was driven down the medial bank of the postlateral gyrus, obliquely crossing a large region of cortex. The first cells encountered in each penetration had receptive fields within 5° of the area centralis and all receptive fields were within 15°.

We studied the responses of neurones to flashing and moving patterns back projected on to a tangent screen 57 cm from the cat's eyes. Receptive fields were classified into one of five groups: orientation selective¹ (simple, complex or hypercomplex), pure direction selective, orientational bias, nonoriented or visually unresponsive⁵. Units with the brisk response, fibre waveform and clear concentric receptive field organisation of afferent fibres from the lateral geniculate nucleus were excluded from the analysis. For all cells that were excited by visual stimuli we assessed the relative responsiveness through the two eyes and classified them using Hubel and Wiesel's seven-point scale of ocular dominance¹. Cells in groups 1 and 7 are solely monocularly driven, group 1 by the eye contralateral to the recording site, group 7 by the ipsilateral eye. Group 4 cells are equally influenced by the two eyes. Groups 3 and 5 are slightly more strongly driven by the contralateral and ipsilateral eyes respectively; groups 2 and 6 are strongly dominated by the contralateral and ipsilateral eyes respectively. All recordings were made from the right hemisphere, contralateral to the initially experienced left eye.

The ocular dominance histograms in Fig. 1a show the results for 27 cells from the control animal, monocularly deprived in the ipsilateral eye until recording at 5 weeks of age, and for 189 cells from six kittens reverse sutured at 5 weeks and allowed various periods of time in which

to use the previously deprived ipsilateral eye. As expected, the first histogram shows that 5 weeks of monocular deprivation silenced virtually all connections from the deprived ipsilateral eye: almost all cells are in group 1. But 3 d after reverse suturing many neurones could be driven by the initially deprived eye. After only 1 week the process was about half complete, and within 3 weeks there was almost no influence from the previously dominant eye. These data are presented graphically in Fig. 1b where the proportion of cells dominated by the initially deprived eye is plotted against the number of days after reverse suturing.

We observed striking changes in the functional architecture of the cortex during reversal of monocular deprivation. Cells were clustered into distinct regions dominated by one eye or the other—the 'ocular dominance columns' of Hubel and Wiesel⁸. We judged the dimensions of these columns by the distances between obvious switches in ocular dominance during each long penetration. The relative size of the columns seemed to change from animal to animal in an ordered manner. In kittens where the two eyes commanded roughly equal numbers of cortical cells, the columns for the two eyes were of about the same size; in the more extreme cases the regions devoted to the eye that, overall, dominated fewer cells were smaller and sparser.

Thus, after reverse suturing, areas dominated by the initially deprived eye appear, then expand and ultimately occupy the whole cortex. Such an orderly anatomical progression suggests that this physiological recapture may represent actual growth and reorganisation of afferent

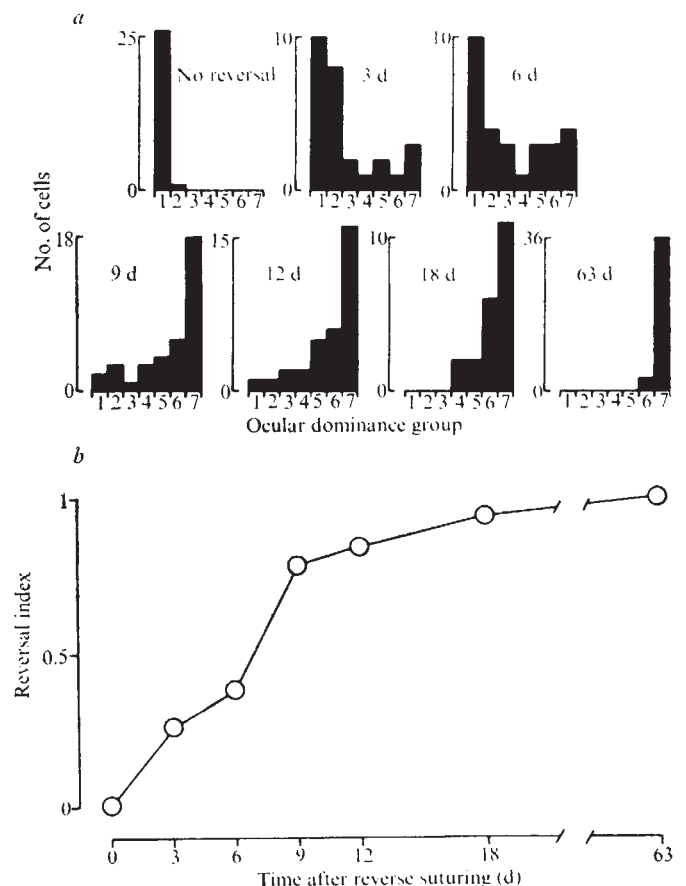


Fig. 1 *a*, The ocular dominance histograms of 216 visually responsive cells from the series of seven kittens recorded at varying times after reversal of lid suture. The ocular dominance classification is described in the text. *b*, The process of reversal is shown graphically, plotting reversal index against days after reverse suturing. The reversal index is calculated by dividing the number of cells dominated by the previously deprived eye (groups 5, 6 and 7) by the total number of cells.

terminals, not merely the strengthening of existing but silent synapses. If so, it implies that the mammalian central nervous system, at least early in life, has some powers of rapid regeneration.

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Intercellular gap junctions in pigment epithelium cells during retinal specification in *Xenopus laevis*

DURING embryonic development the cells of the retina acquire positional information which predisposes the future optic axons to connect with specific loci in the optic tectum. In *Xenopus* embryos the eye becomes irreversibly polarised along the naso-temporal and dorso-ventral axes by stage 32¹, after which it is insensitive to any specifying influence previously exercised by the tissues surrounding the eye².

In a recent electron microscope study³ of the developing *Xenopus* retina we described the fine structure of the developing retina between stages 26 and 36. We noted then, the presence of intercellular gap junctions which occurred throughout the retina up to stage 30/31. Beyond stage 32 these junctions could only be found at the periphery of the retina where stem cells contribute new cells to the growing retina⁴. Hence the disappearance of these gap junctions from the central portion of the developing retina appears to be correlated with the time of retinal specification.

We have recently re-examined our data on the developing *Xenopus* eye and noted a significant feature which we failed to report in our original paper. Namely, that cells of the embryonic pigment epithelium, as well as cells of the neural retina, establish gap junctions with one another throughout the eye prior to stage 30/31. We also observed gap junctions between retina and pigment epithelium (Fig. 1). After stage 32 these gap junctions disappear from the centre of the eye but persist for some time in the periphery.

Many results support the view that the flow of molecules through cell junctions may be important in the control of cellular growth and differentiation⁵. In our previous paper we suggested that the disappearance of gap junctions from the central part of the eye and the time of retinal determination might be causally related. Our present observations on the distribution of gap junctions between developing retina and pigment epithelium prior to stage 32 suggests that not

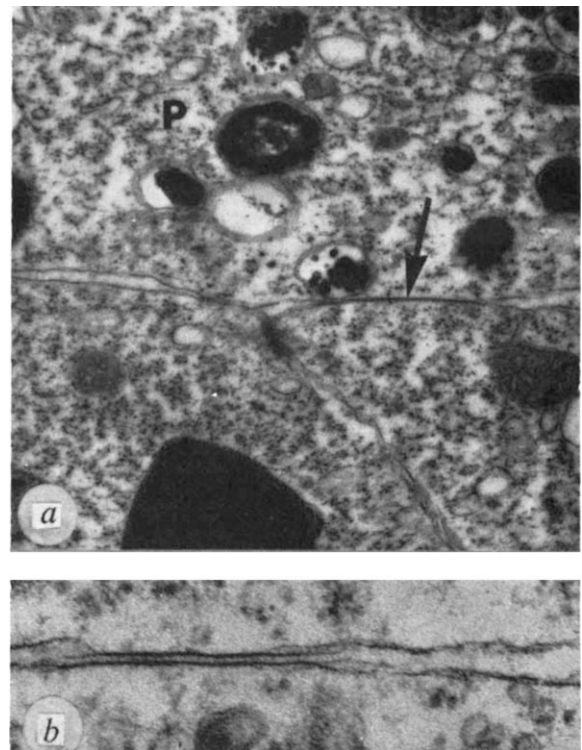


Fig. 1 *a*, The arrow indicates an intercellular junction between a germinal pigment epithelial cell (P) and a retinal cell at stage 26. ($\times 24,000$); *b*, A similar junction at higher power illustrating the characteristic narrow gap of 3-9 nm. ($\times 94,000$).

only the retina, but also the pigment epithelium, may be subject to the specifying influence of the surround through intercellular channels. This is particularly interesting in view of recent indications that pigment epithelium cells from the centre of the eye of an adult newt may pass on positional information to a new regenerating retina⁶.

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Specificity of regenerating optic fibres for left and right optic tecta in goldfish

RECENT studies¹ on the regeneration and development of the visual nervous system of inframammalian vertebrates have concentrated on the topographic patterning of retinotectal connections, neglecting the problem of what specifies the pathways of large tracts. Embryologically the eye is an outgrowth from the ipsilateral side of the brain, but during development the optic nerve fibres cross the midline and terminate on

the opposite side where they "mirror" the topographical disposition of their retinal ganglion cells. Sperry^{2,3} proposed that the precise patterning of such neuronal circuits resulted from specific affinities between individual nerve fibres and their target loci within the brain, but recent evidence⁴ suggests that what is important is the preservation of the overall pattern of fibre connections, rather than the position occupied by individual terminals⁵.

Investigation of the pattern of recovery following reinnervation of muscles in fish^{6,7} suggests that competitive interaction between different nerve fibres may in some species lead to the selection of correct connections. We now report some preliminary results where both optic nerves in goldfish were directed into the same tectum to test for competitive interaction for occupancy of the same tectal site.

Goldfish were anaesthetised in a 0.4% solution of MS222 (Sandoz), and mounted in a holder which allowed the gills to be continuously perfused with water, or with a solution of MS222, as required. Two operations (Fig. 1) were then performed on the fish. The tectum of the anaesthetised fish was exposed by opening a cranial bone flap which was replaced after surgery. A small tungsten wire hook was then inserted under the left optic tract, optic fibres stripped away from the left tectum, and the length of nerve brought right over to lie

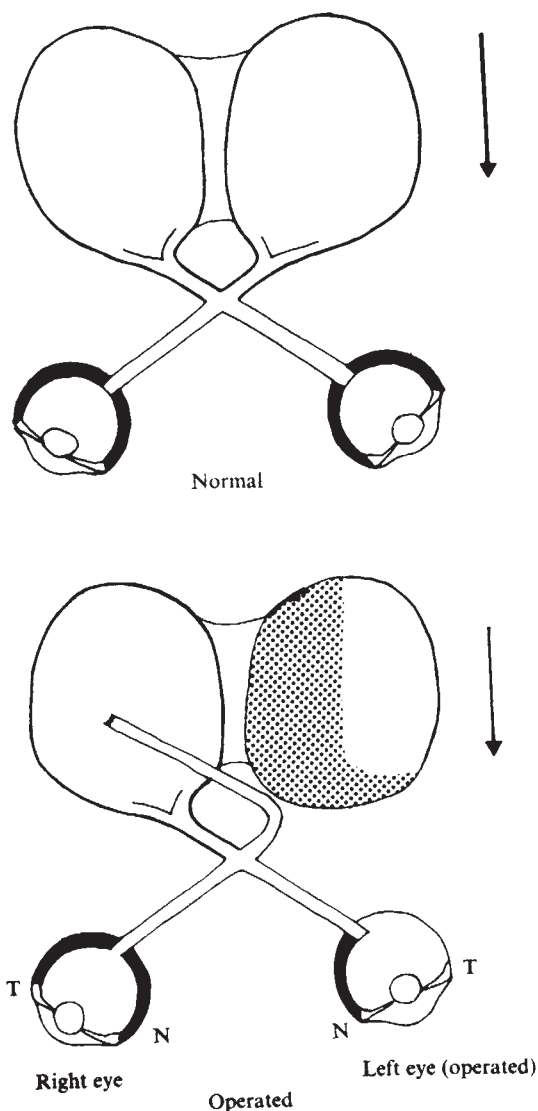


Fig. 1 Operations. The optic nerve from the right eye was uncrossed surgically and diverted to the right tectum, and the temporal half of the retina from the left eye was removed. Shaded region shows area of tectum removed.

across the right optic tectum. A medial strip from the left tectum was excised by aspiration along its whole length to prevent the nerve from growing back to the original tectum and the bone flap replaced. A second operation was then performed to remove the temporal hemiretina from the fish's left eye (other experiments where a hemiretina was removed from each eye will be reported later). In each case an incision was made to remove the hemiretina by cutting along the vertical meridian at the back of the eye. Bleeding was reduced by a noose of silk thread slipped around the optic nerve. After the operation the fish were returned to individual home tanks where they were kept for a year before recording. The length of the postoperative period was to allow any possible neuronal respecification⁸ to occur before investigation.

For electrophysiological recording anaesthetised fish were mounted in their holder, and the tectum exposed. One eye was centred on a projection perimeter (the other eye was always covered with a plasticine occluder) and the visuotectal representation for that eye on the right tectum mapped with a Woods-metal microelectrode⁹. The same procedure was then repeated for the other eye.

In 21 of the 24 fish which survived the recovery period, the nerve from the right eye failed to form connections with the ipsilateral tectum and showed a marked tendency to curve back to try and reach the remaining fragment of lateral tectum on the contralateral (and correct) side. Unfortunately the left tectal fragment was often placed too far laterally to enable us to record from it effectively. In three fish, where the operation was successful, one was a control, where in addition to uncrossing the chiasma we removed the nasal hemiretina from the left eye and the temporal hemiretina from the right eye. In this control fish, the representation of the hemiretinas from the right

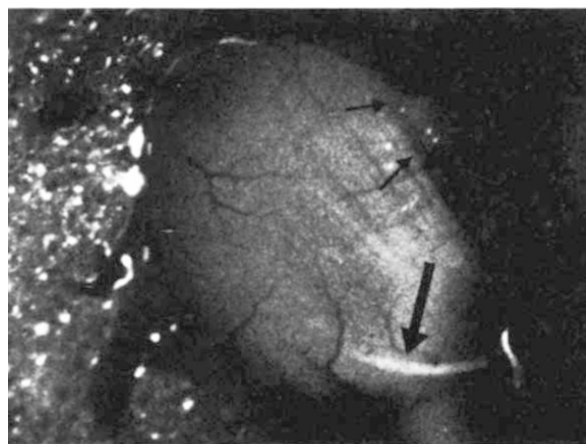


Fig. 2 Large arrow: insertion of the uncrossed optic nerve from the right eye into the ipsilateral optic tectum. A band of newly myelinated fibres can just be seen in the caudal region of the tecto-mesencephalic commissure (small arrows).

and left eyes, were each confined to their 'correct' half of the right tectum. The other two fish gave results that were similar to one another. Figure 2 shows the tectum of one of them, where we succeeded in getting the optic nerve from the right eye (or at least part of it) to deploy itself in the ipsilateral optic tectum. There was also some suggestion that optic fibres from the right temporal hemiretina had crossed back to the correct (left) tectum via the tecto-mesencephalic commissure. Indeed, a band of what appeared to be newly myelinated fibres was clearly visible at the caudal end of this commissure (Fig. 2). Furthermore, on the right optic tectum, we found no evidence, electrophysiologically, of any representation of the temporal retina from the right eye (Fig. 3). Two zones innervated exclusively by one eye or the other are separated by a zone in which the two projections overlap. The area of overlap seemed to vary in different fish, and it is possible that during the early stages of recovery the two projections may have overlapped completely and only later become segregated from one another.

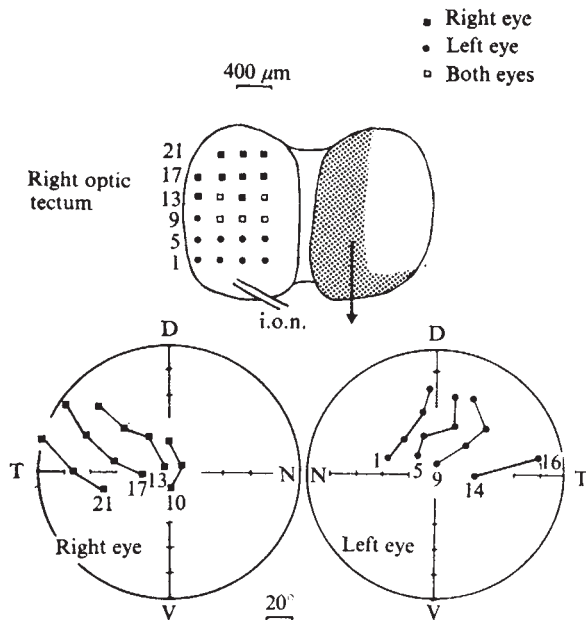


Fig. 3 Visual field projections from the right and left eyes onto the right optic tectum. Shaded region shows area of tectum removed. i.o.n.; optic tract from ipsilateral eye.

Previous studies¹⁰ have shown that if a sufficient period is allowed for recovery, the fibres from a nasal hemiretina can spread and occupy a whole normal tectum. By contrast, in Fig. 3 the projection from the left nasal hemiretina only extends over approximately half the dorsal surface of the tectum. Moreover, in this instance the nerve from the contralateral eye occupied the wrong half of the tectum (in the normal contralateral projection¹¹ optic fibres from the nasal hemiretina occupy the caudal half of the tectum). This suggests that the contralateral nerve fibres may have been displaced forwards by competition for tectal sites with fibres arriving from the ipsilateral nasal hemiretina.

While it is clearly too early to draw any hard and fast conclusions, our observations indicate (1) that regenerating optic fibres may be able to distinguish between right and left tecta, and (2) if both optic nerves are forced into the same tectal field, each may have a tendency to exclude the other from the tectal zone it innervates.

There is one further point worth noting. Yoon⁸ found that in certain conditions, goldfish optic fibre terminals would arrange themselves topographically in an anomalous region of the optic tectum. Yoon attempted to reconcile his results with Sperry's original chemoaffinity hypothesis³ by suggesting that respecification of tectal elements might have occurred after surgery. Our study, however, makes this interpretation unlikely. While only the nasal half of each retina was represented on the tectum, optic fibres from corresponding retinal loci deployed themselves topographically at different locations of the same unoperated tectum. It is difficult to envisage how respecification of tectal elements could account for the development of a duplicate set of 'tectal markers' in an unoperated optic tectum.

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Evidence for two mechanisms of sclerotisation in insect cuticle

IN many insects the cuticle changes drastically in properties soon after a moult. When the insect emerges from the old cuticle the new one is soft, pale, and a major part of the proteins can easily be extracted. Gradually the proteins become insoluble, the cuticle becomes harder, and in many cases it also becomes darker. The colour may range from a very light amber to dark brown or even black. It has been discussed repeatedly whether hardening and darkening are two independent processes or whether they are two results of the same chemical reaction, but no definitive conclusions have been reached¹⁻⁶.

During a study of cuticle sclerotisation I have obtained evidence which confirms an earlier suggestion⁷ that two mechanisms for forming cross links exist in insect cuticle, one giving a colourless cuticle and the other a dark brown cuticle. Both mechanisms can be active simultaneously in the same piece of cuticle and they can utilise the same substrate, N-acetyldopamine. In the reaction resulting in a colourless cuticle the β position of the substrate is activated and becomes involved in cross linking of the proteins, while the aromatic ring is involved in the reaction giving rise to a brown cuticle.

Both activities were assayed by means of tritiated N-acetyldopamine, for the former assay tritium was located on the β carbon atom of the sidechain and for the latter assay it was restricted to the aromatic ring of the substrate. The two substrates were synthesised by treatment of 3,4-dihydroxyphenylethylamine (2^3H^- ethyl) and 3,4-dihydroxyphenylethylamine (3^3H^- ring), respectively, with acetic anhydride in 10% potassium tetraborate⁸. The purified products were diluted with unlabelled N-acetyldopamine to give 10 mM solutions which contained 2 $\mu\text{Ci ml}^{-1}$.

Samples of cuticle were obtained from the desert locust, *Schistocerca gregaria*, the silk moth, *Hyalophora cecropia*, and from meal worms, *Tenebrio molitor*. All cuticles were carefully cleaned and washed in 1% potassium tetraborate and in distilled water. They were divided into the appropriate parts which were incubated in 2.6 ml 0.2 M Na-acetate buffer, pH 5.5, 100 μl of either one or the other substrate was added, and the mixture was incubated for 18 h at room temperature. The tritium which was split off during this period was determined after passing the supernatant through a small column (0.5 $\times$ 1 cm) of activated charcoal, which retains the aromatic substrate but not tritiated water⁸. The first millilitre of the eluate was discarded, and from the rest of the eluate 1 ml was taken to be counted. The non-enzymatic release of tritium from the substrates was determined in parallel experiments without any cuticle and was used to correct the experimental results. The dry weight of the cuticular samples was determined after the incubations by drying them to constant weight at 100°C.

Table 1 shows that all the cuticular samples will release tritium from both substrates and that the relative activities vary considerably. The various types of locust cuticle which remain lightly coloured during sclerotisation show a much higher activity towards the sidechain of N-acetyldopamine than towards the ring, whereas the mandibles, which are a

Table 1 Amounts of tritium released from ring- ^3H -labelled and β - ^3H -labelled N-acetyldopamine (NADA) on incubation with various cuticular samples

Sample	Colour of fully hardened cuticle	n*	Radioactivity (c.p.m. $\text{mg}^{-1} \pm \text{s.d.}$) released from		Ratio ring- $^3\text{H}/\beta$ - ^3H
			ring- ^3H -NADA	β - ^3H -NADA	
<i>Schistocerca gregaria</i>					
Adults					
Femur	Colourless with black areas	4	438 $\pm$ 136	7,590 $\pm$ 1398	0.06
Tibia	Colourless with dark brown spines	4	1,121 $\pm$ 190	7,733 $\pm$ 1237	0.15
Lateral meso- and metathorax	Colourless with dark ribs	4	571 $\pm$ 73	10,052 $\pm$ 1250	0.06
Dorsal meso- and metathorax	Light brown	2	1,090	13,126	0.08
Ventral meso- and metathorax	Colourless	2	491	6,106	0.08
Pronotum	Colourless	2	671	10,098	0.07
Dorsal abdomen	Colourless	4	233 $\pm$ 105	11,400 $\pm$ 254	0.02
Ventral abdomen	Colourless	2	107	7,789	0.01
Head capsule	Colourless	4	429 $\pm$ 112	9,990 $\pm$ 1110	0.04
Mandibles	Dark brown	2	1,443	3,000	0.48
Fifth instar larvae					
Femur	Colourless with black areas	5	2,359 $\pm$ 123	33,052 $\pm$ 620	0.07
Mandibles	Dark brown	3	4,411	4,474	0.99
<i>Tenebrio molitor</i>					
Adults					
Ventral abdomen, young	Dark brown	6	6,064 $\pm$ 714	4,005 $\pm$ 780	1.51
Ventral abdomen, old	Dark brown	2	272	2,488	0.11
<i>Hyalophora cecropia</i>					
Larval abdomen	Colourless	6	1,888 $\pm$ 109	6,504 $\pm$ 104	0.29
Young pupae	Dark brown	4	13,177 $\pm$ 1808	14,404 $\pm$ 1390	0.91
Old pupae	Dark brown	4	10,225 $\pm$ 1375	18,881 $\pm$ 1331	0.54

The adult locusts were killed 1 d after the moult, their cuticle was thus well sclerotised, while the fifth instar larvae were caught while moulting. The young *Tenebrio* adults were caught less than 12 h after the moult and were light brown in colour, whereas the old adults were about 2 week old and were very darkly coloured. The young *Hyalophora* pupae had an uncoloured frontal part and a light brown abdomen, and the old pupae were dark brown all over.

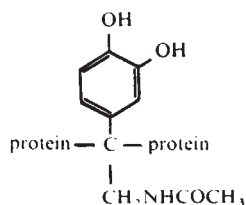
* Number of determinations.

dark brown type of cuticle have a relatively higher activity towards the ring part of the substrate. No significant differences are observed between those samples of locust cuticle which become melanised and those which do not.

The pupal cuticle of *Hyalophora*, which becomes dark brown during hardening, contains high levels of both activities, whereas the enzyme active towards the sidechain of the substrate dominates in the colourless larval cuticle. In young *Tenebrio*, where only little sclerotisation has occurred, the enzyme active towards the aromatic ring dominates slightly, but this activity disappears as the cuticle becomes fully sclerotised, and the sidechain directed activity remains.

The results indicate that cuticles which become dark brown during sclerotisation contain relatively large amounts of an enzyme which is active towards the ring moiety of N-acetyldopamine, whereas cuticles which remain pale or colourless prefer the sidechain of the substrate.

We have in some earlier publications reported on the sclerotisation of locust cuticle⁷⁻⁹, and our results suggested that the cross links in hard, uncoloured cuticle are:



It can be assumed that the enzyme which can remove tritium from the β position of N-acetyldopamine is also responsible for the formation of the cross links⁸. The enzyme which removes tritium from the aromatic ring can be assumed to be an *o*-diphenoloxidase oxidising the substrate to an *o*-quinone which will combine with free amino groups with the simultaneous loss of tritium from the ring. This enzyme would thus be responsible for the classical type of quinone tanning of insect cuticle first suggested by Prior¹.

The possibility that both enzyme activities reside in a single enzyme cannot be completely disregarded, although it seems

difficult to explain how the ratio between the two activities can vary so much between the various types of cuticle if it is not two different enzymes. The decrease of only one of the activities as sclerotisation progresses in *Tenebrio* cuticle also indicates that it is two enzymes. It will, however, be necessary to obtain both activities in soluble form before this problem can be solved.

The results reported here indicate that insect cuticle contains two enzyme activities, both involved in sclerotisation and both utilising the same substrate, but one of them resulting in a hard and colourless cuticle and the other giving rise to a hard and dark cuticle. The intensity of the colour will presumably be determined by the relative amounts of the two enzymes in the cuticle and by the possible deposition of melanins, for which a third enzyme can be assumed to be responsible.

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Hepatic necrosis caused by furosemide

FUROSEMIDE (frusemide) is a diuretic drug frequently used in the treatment of many cardiovascular and renal diseases. Its use is contraindicated in pregnancy because of its recognised teratogenic potential¹. The drug also has been reported to potentiate renal injury when used in combina-

tion with cephaloridine^{2,3}. Here we report that this widely prescribed therapeutic agent is converted by microsomal enzymes in the liver of mice and humans to a reactive arylating metabolite that produces massive hepatic necrosis in mice.

Male, Swiss albino mice (20–25 g, National Institutes of Health stock) were given furosemide (400 mg kg⁻¹, intraperitoneally, in 0.9% saline solution) and killed at various intervals. Paraffin sections of the liver were prepared and stained with haematoxylin and eosin.

Within 3 h of furosemide administration, nucleolar changes were present in midzonal hepatocytes, and single cell necrosis with pyknotic hepatocytes showing eosinophilic degeneration was also occasionally present. By 6 h, necrosis of many hepatocytes was found in the midzonal and centrilobular areas, demonstrated by nuclear pyknosis, karyorrhexis and cytoplasmic eosinophilia (Fig. 1a). Early polymorphonuclear cell infiltration was often present in the less severely injured livers. By 12 h, severely damaged livers showed massive necrosis in both the midzonal and the centrilobular areas such that confluent zones of pyknotic, eosinophilic hepatocytes bridged adjacent lobules (Fig. 1b). The Küpffer cells, however, were generally normal. At later time intervals, the pattern of necrosis was usually centrilobular although occasional sections had entirely midzonal necrosis with a collar of viable parenchymal cells remaining around the centrilobular and periportal veins. Necrosis was greatest at 24–48 h; regeneration was evident by 48–72 h.

The histological slides were coded and the severity of the liver necrosis produced by furosemide was determined by two observers blind to the code, as previously described for other hepatotoxins⁴. Furosemide-induced hepatic necrosis was dose dependent (Table 1). After a single furosemide dose of 200 mg kg⁻¹, only slight necrosis (+) was observed in 16% of animals. After administration of 300 mg kg⁻¹, necrosis was present in 68% of mice and was more extensive (++ and +++). After 400 mg kg⁻¹, 92% of mice had necrotic livers and the severity was (++ to +++) in 60% of the animals.

Table 1 Extent of hepatic necrosis in mice 24 h after various intraperitoneal doses of furosemide

Dose of furosemide (mg kg ⁻¹)	No. of animals	% Mortality	Extent of necrosis*				
			O	+	++	+++	++++
100	25	0	100	0	0	0	0
200	25	0	84	16	0	0	0
300	50	2	32	48	16	4	0
400	50	4	8	32	36	18	6
500	25	20	0	5	40	35	20

* Extent of necrosis (determined as previously described⁴): O, absent; +, necrosis of less than 6% of parenchymal hepatocytes; ++, necrosis of 6–25% of hepatocytes; +++, 26–50% of hepatocytes; +++++, greater than 50% of hepatocytes.

Alterations in hepatic blood flow with resulting hypoxia may play a role in the pathogenesis of centrilobular liver damage. Therefore, we considered the possibility that the hepatotoxicity produced by furosemide might have been caused by hypoxia resulting from massive diuresis with subsequent decrease in the vascular volume. Furosemide-treated mice were never in shock, however, nor showed respiratory depression during our studies, and other diuretics, such as chlorothiazide and ethacrynic acid, did not cause hepatic necrosis even after LD₅₀ doses. Moreover, the dose of furosemide required to produce necrosis was much greater than that which usually produces maximal diuresis⁵ and post-treatment with Ringer's lactate in 5% glucose (12.5 ml kg⁻¹, intraperitoneally, at 1, 2, 3 and 4 h after

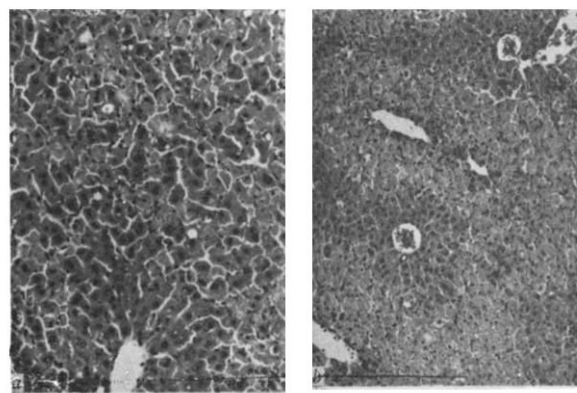


Fig. 1 Furosemide-induced liver necrosis in mice after an intraperitoneal injection of 400 mg kg⁻¹ of furosemide. Sections of liver stained with haematoxylin and eosin. a, Midzonal hepatic necrosis 6 h after furosemide ($\times 98$); b, centrilobular-midzonal hepatic necrosis 12 h after furosemide ($\times 44$).

administration of furosemide) did not prevent the lesion. In addition, the initial hepatic lesion occurred midzonally, not centrilobularly, and was present a few hours after drug administration. Finally, the liver damage is probably caused by a metabolite of furosemide rather than the parent drug, because necrosis was prevented when the metabolism of furosemide was inhibited by pretreatment of mice with three different types of cytochrome P-450 enzyme inhibitors: piperonyl butoxide, cobaltous chloride and α -naphthylisothiocyanate.

In a further series of experiments we studied the enzyme-dependent covalent binding of furosemide to hepatic macromolecules both *in vitro*³ and *in vivo*⁶; this technique can be used as an index of the formation of an arylating metabolite of a drug^{7,8}. Covalent binding *in vitro* of ³H-furosemide (generally labelled) or ³⁵S-furosemide to hepatic microsomal protein required oxygen and a NADPH generating system and was inhibited by an antibody against NADPH-cytochrome c reductase and by a 9:1 carbon monoxide:oxygen atmosphere. Thus, the formation of an arylating metabolite of furosemide was mediated by a cytochrome P-450-dependent mixed-function oxidase in liver. We also found a furosemide metabolite bound to hepatic protein *in vivo* to about the same extent (1.5 nmol mg⁻¹ protein) as we had previously found for the reactive, hepatotoxic metabolite of acetaminophen (paracetamol)⁷.

Evidence of the covalent nature of the furosemide binding was obtained by hydrolysis with pronase of the solvent-extracted protein precipitate and isolation of the radio-labelled compound bound to single amino acids and peptide fragments. Pretreatment of mice with piperonyl butoxide, cobaltous chloride or α -naphthylisothiocyanate almost completely abolished both the *in vivo* covalent binding of furosemide and furosemide-induced hepatic necrosis. The covalent binding occurred a few hours before the onset of histologically recognisable necrosis and before biochemical changes in the hepatocytes (such as, decreased cytochrome P-450-dependent drug metabolism and decreased protein synthesis). These findings lead us to conclude that the arylation of liver macromolecules by a reactive furosemide metabolite is causally related to the development of furosemide-induced hepatic necrosis.

Another important finding was the presence of a dose threshold for the covalent binding and liver necrosis produced by furosemide⁴. Only small amounts of covalent binding and no necrosis occurred until a dose of 100 mg kg⁻¹ was exceeded. Studies on the metabolism, distribution and reversible plasma protein binding of furosemide after non-toxic and toxic doses suggest that this threshold may result

from a saturation of the organic anion binding sites on plasma proteins after toxic doses of furosemide⁸. More free furosemide would then be available to the liver for metabolism. Unlike the dose threshold for acetaminophen-induced liver necrosis¹⁰, the furosemide threshold is not the result of a protective role of glutathione since furosemide did not deplete hepatic glutathione.

The hepatic injury produced by furosemide (N-(2'-furyl-methyl)-4-chloro-5-sulphamoylanthranilic acid) apparently results from the metabolic activation of the furan ring, possibly by an epoxidation similar to that proposed for the hepatocarcinogenic dihydrofuran aflatoxins¹¹. For example, furosemide radiolabelled with tritium in its furan moiety was bound covalently to hepatic microsomes in the presence of oxygen and NADPH to the same extent as furosemide radiolabelled specifically with ³⁵S in its sulphonamide moiety, demonstrating that the bound metabolite contained both parts of the furosemide molecule. To determine where the binding occurred on the molecule, the metabolite-protein conjugates isolated from the liver were hydrolysed in mild acid conditions (pH 4.5) that split furosemide into its methylfuran and sulphamoylanthranilic acid portions. The binding of furan-radiolabelled furosemide to protein was unchanged whereas the binding of ³⁵S-labelled furosemide was lost. Thus, the metabolic activation of furosemide must have occurred on the furan ring.

Finally, we have compared the hepatotoxicity of structural analogues of furosemide and have shown that the hepatic lesion can be reproduced by such simple furans as 2-hydroxymethylfuran, 2-acetylfuran and even furan itself (Table 2). Moreover, pretreatment with phenobarbital shifts the zone of necrosis produced by furosemide and by other furan-containing compounds such as ngaione and 3-hydroxymethyl furan (N,N-diethyl)-carbamate^{12,13} from a centrilobular to midzonal distribution in mice. Furosemide, furan, 2,3-benzofuran and certain other furano compounds cause toxic lesions in the kidney (Table 2) in addition to the liver, whereas ipomeanol and other furan analogues selectively produce lung damage and pulmonary oedema^{14,15}. Thus, a variety of tissue lesions seen after furan-containing compounds may result from a metabolic activation similar to that proposed for furosemide. Extension of these studies to thiophene, an analogue of furan in which the oxygen atom in the five-membered ring is replaced with sulphur, has shown that several thiophene-containing compounds also produce massive hepatic or renal necroses in animals (J.R.M., W.Z.P., J.A.H. and D.J.J. unpublished). Studies are underway to determine whether the nephrotoxicity produced in humans by cephaloridine and cephalothin results from the metabolic activation of the thiophene nucleus in these antibiotic drugs.

The implications of the present findings for the clinical use of furosemide are uncertain. Extensive clinical experience with furosemide has not resulted in reports of liver damage; presumably therefore, the drug is not hepatotoxic at the usual therapeutic doses (40–600 mg d⁻¹). The enzyme pathway responsible for the formation of the toxic metabolite, however, is apparently present in humans because human cadaver microsomes, similar to mouse microsomes, can convert furosemide to an arylating metabolite (J.R.M., W.Z.P., J.A.H., and D.J.J., unpublished). The possibility arises, therefore, that the remarkable safety of furosemide after low therapeutic doses may result from the presence in humans of a dose-threshold phenomenon similar to that found in mice. If this is so, then tissue concentrations of furosemide in patients with renal failure probably are much greater in relation to the furosemide dose, since furosemide is eliminated from the body primarily by renal excretion of the unchanged drug¹⁶. In addition, patients with chronic renal failure often have decreased concentrations of plasma proteins, making more free (nonbound) furosemide available to the liver for metabolism. Since huge doses of furose-

Table 2 Liver, kidney and lung toxicity in mice and rats after furan analogues, given intraperitoneally.

Liver*	Dose† (mmol kg ⁻¹)	Kidney*	Dose† (mmol kg ⁻¹)	Lung*
Furan	3.5	Furan	3.5	Ipomeanol§
Furosemide	1.1	2-ethyl furan	2.4	
2-Furamide	0.7	2, 3-benzofuran	1.3	
2-Acetyl furan	2.0	2-furoic acid	1.9	
2-Furfural	3.9			
2-Ethyl furoate	3.6			
2-Methoxy furan	8.4			
Dibenzofuran	5.8			
Ngaione‡				

* Primary site of lesion. However, the other organs frequently manifested damage but to a lesser degree. Moreover, the site of the lesion often was shifted from one organ to another by pretreatments that altered drug metabolism, for example, administration of phenobarbital or piperonyl butoxide (unpublished results).

† Dose producing necrosis of 6–25% of parenchymal hepatocytes or 6–25% of cells of renal convoluted tubules.

‡ Data from ref. 13.

§ Data from ref. 14.

mide (for example 2,000 mg by rapid intravenous injection) are currently used to treat patients with acute and chronic renal failure¹⁷, the potential hepatotoxicity of this therapeutic regimen should be carefully evaluated. The possible consequences of the formation in humans of an arylating metabolite from furosemide should also be considered, because most compounds that arylate tissue macromolecules *in vivo* can produce neoplasia¹⁸.

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Red cell 2,3-diphosphoglycerate concentration in man decreases with age

So far there has been no report in the literature suggesting that the chemical composition of the red blood cell changes during the life of an adult healthy human. We have now obtained evidence that in a normal population the red cell concentration of 2,3-diphosphoglycerate (2,3-DPG) decreases with advancing age. 2,3-DPG is an intermediary metabolite in the Embden-Meyerhof glycolytic pathway in the red cells, which affects haemoglobin affinity for oxygen. Elevated concentration of 2,3-DPG decreases the affinity and thus increases the fraction of haemoglobin-bound oxygen available to the tissues, and decreased concentration of 2,3-DPG has the opposite effect^{1,2}. In general, an increase in the red cell 2,3-DPG is found in response to hypoxia or anaemia and a decrease of 2,3-DPG is caused by acidosis^{3,4}.

with advancing age, and the difference in concentrations between males and females persisted in all age groups.

It is possible that the decrease of 2,3-DPG in the red cell is related to lower concentrations of plasma inorganic phosphate found in old age⁶. This is supported by observations that the red cell 2,3-DPG changes in parallel to the concentration of plasma inorganic phosphate in conditions with hyperphosphataemia⁷ and hypophosphataemia^{8,9}. Recently, increased concentrations of 2,3-DPG and plasma inorganic phosphate were found in children between 1 and 12 yr of age¹⁰. These children, however, had significantly lower than normal haemoglobin concentrations. Whether in children the 2,3-DPG concentration is increased in response to high plasma inorganic phosphate concentrations or to reduced haemoglobin is not clear.

The physiological significance of the decreased red cell 2,3-DPG in the elderly is uncertain. We calculated, on the basis of results obtained by other workers in transfused postoperative patients^{11,12}, that in the octogenarians studied by us the decreased 2,3-DPG would on average shift the p_{50} of the oxygen dissociation curve of haemoglobin 2 mm Hg to the left; the corresponding change in the delivery of oxygen to the tissues would only be slight. On the other hand, Birnstingl *et al.*¹³ found considerably higher haemoglobin affinity for oxygen in normal men over 40 yr of age as compared to those younger than 40, when they measured oxyhaemoglobin dissociation at

Table 1 Relationship between age and concentrations of red cell 2,3-diphosphoglycerate (2,3-DPG) and haemoglobin in 'normal' population

Age (yr)	2,3-DPG* ($\mu\text{mol per g haemoglobin}$)			Haemoglobin (g per 100 ml)		
	No.	All subjects Mean $\pm$ s.d.	No.	Males Mean $\pm$ s.d.	No.	Females Mean $\pm$ s.d.
18-24	26	14.9 $\pm$ 1.6	36	15.0 $\pm$ 1.0	17	13.5 $\pm$ 0.8
25-34	35	14.4 $\pm$ 1.3	36	15.4 $\pm$ 0.6	8	14.0 $\pm$ 0.9
35-44	17	14.3 $\pm$ 1.5	14	15.6 $\pm$ 0.6	7	13.6 $\pm$ 1.0
45-54	17	14.2 $\pm$ 1.5	8	14.6 $\pm$ 0.3	9	14.1 $\pm$ 0.9
55-64	11	14.9 $\pm$ 1.8	6	15.1 $\pm$ 0.3	5	14.1 $\pm$ 1.9
65-74	56	13.8 $\pm$ 1.9†	26	15.3 $\pm$ 1.3	30	13.9 $\pm$ 0.8
75-84	104	13.9 $\pm$ 2.4†	46	14.8 $\pm$ 0.9	58	13.9 $\pm$ 0.8
85 and over	16	12.8 $\pm$ 2.0†	4	15.1 $\pm$ 0.8	12	14.1 $\pm$ 1.1

* Regression equation for individual values of 2,3-DPG and age: $y = -0.02x + 15.25$; $r = -0.2036$ ($P < 0.01$).

† Significantly lower than for Group 18-24 yr of age ($P < 0.05$; Student's t test).

None of the subjects were ill or seeking medical help at the time of investigation. There were no heavy smokers (more than 20 cigarettes in a day). None of the subjects were taking iron, folate or vitamin B₁₂ supplements. Excluded from the study were the subjects in whom anaemia (haemoglobin concentration < 13.5 g per 100 ml for males and < 11.5 g per 100 ml for females) and/or deficiency of iron (serum iron concentration < 65 $\mu\text{g per 100 ml}$ and TIBC > 410 $\mu\text{g per 100 ml}$), folate (serum and red cell folate concentrations < 4.0 ng ml⁻¹ and < 160 ng ml⁻¹, respectively) or vitamin B₁₂ (serum vitamin B₁₂ concentration < 160 pg ml⁻¹) were found. Blood counts were determined using a Coulter Counter Model S. 2,3-DPG concentration was measured in 1:400 aqueous dilutions of heparinised or sequestrenated blood by a modified automated method of Grisolia *et al.*¹⁴ using an Auto-Analyzer (Technicon). The concentrations of iron, folate and vitamin B₁₂ in blood were determined using standard colorimetric and microbiological assays.

A total of 322 subjects (176 males and 146 females) from 18 to 97 yr of age were investigated. They were selected from the laboratory staff and the participants of the Nutritional Survey of the Elderly, carried out by the Department of Health and Social Security⁵, by excluding those in whom anaemia and/or deficiency of iron, folate or vitamin B₁₂ were found.

When subjects were grouped according to age a progressive decrease of 2,3-DPG concentration with advancing age was found (Table 1). The decrease from the mean 2,3-DPG of 14.9 $\mu\text{mol per g haemoglobin}$ for the first group (18 to 24 yr) became statistically significant ($P < 0.05$) for the groups comprising subjects older than 65 yr. Furthermore, a significant ($P < 0.01$) inverse correlation between 2,3-DPG concentration and age was found when individual values were correlated by regression analysis. Within each group the mean 2,3-DPG concentrations in males and females were essentially the same, except in the age group 35 to 44 yr, where the difference between mean concentrations for males and females (13.6 and 15.2 $\mu\text{mol per g haemoglobin}$, respectively) was significant ($P < 0.05$). There was virtually no change in haemoglobin concentration

p_{O_2} of 9 and 13 mm Hg. Furthermore results from his laboratory indicate that in some anaemic subjects over 65 yr of age, the increase of 2,3-DPG was less than expected for the given degree of anaemia. Both latter studies would therefore indicate that at least in some elderly subjects decreased red cell 2,3-DPG may impair the mechanism of adaption to hypoxia or anaemia.

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Glucose accumulation by rat small intestine during absorption *in vivo*

GLUCOSE is transported across the mammalian small intestine even when its concentration in the lumen is lower than that in the plasma. According to the hypothesis of Crane¹, this 'active transport' is coupled to the movement of sodium ions, which are maintained at a lower concentration in the mucosal epithelium of the gut than in the lumen. Glucose and sodium are thought to combine with a 'carrier' molecule at some external site on the brush border of the epithelial cells, the glucose then moving uphill into the cell at the expense of the movement of sodium down its electrochemical potential gradient². The concentration of glucose in the mucosa rises to a level above that in the blood capillaries at the base of the epithelium, so that the sugar can move downhill into the plasma. The accumulation of glucose to give a tissue concentration higher than that in the bathing fluid has repeatedly been

observed in a wide variety of *in vitro* intestinal preparations and is here extended to an *in vivo* situation.

About 20 cm of jejunum from male Wistar rats (200–250 g) was exposed under ether anaesthesia, and the lumen gently rinsed with 10 ml ice cold 0.9% NaCl. The segment was cannulated and the lumen perfused, *in situ* (that is, with intact mesentery), with Krebs-Ringer bicarbonate medium, in a gas lift, recirculation apparatus, similar to that described by Fisher and Parsons³. The gas mixture was 95% O₂ and 5% CO₂. During perfusion, the gut was replaced inside the body wall, and the animal maintained continuously under ether. (It is likely that the relatively high blood glucose levels shown in Table 1 were due to the deleterious effects of the ether anaesthesia.) After 5 min perfusion, glucose was added to the luminal circuit, and the perfusion continued for a further 10 min. After this time, a sample of perfusion medium was taken, the gut re-exposed, and a portion, approximately 4 cm long, immediately clamped between aluminium tongs precooled in liquid nitrogen⁴. The frozen segment was kept in liquid nitrogen while approximately 1 ml blood was withdrawn from the heart into heparinised tubes.

The final (that is, 15 min) concentrations of glucose in the whole gut wall (after correction for contaminating luminal and blood glucose), in the luminal fluid, and in the blood plasma, are shown, for a variety of experimental conditions in Table 1. The tissue to lumen, and tissue to plasma final concentrations ratios of glucose are also included in Table 1.

When the initial concentration of glucose in the lumen was 5.55 mM, there was an approximately two-fold uphill gradient of glucose from lumen to plasma. In this situation, the gut wall concentrated the glucose to a level almost 1.5 times that in the plasma, and over three times that in the lumen, so that a downhill movement of glucose could take place from the tissue into the blood. Phlorizin, a specific inhibitor of glucose active transport reduced the accumulation of glucose by the tissue (tissue:plasma ratio 0.90). With an initial luminal glucose concentration of 28 mM, there was a downhill gradient of glucose from lumen to plasma, so that concentration of the glucose by the tissue to a level above that in the plasma was no longer necessary, and was not observed. These findings are in accord with Crane's hypothesis, but they differ from that of Esposito *et al.*⁷ whose technique, however, perhaps allowed loss of

Table 1 Glucose concentrations in luminal fluid, blood plasma, and gut wall

Treatment	No. of observations	Lumen concentration Initial	Lumen concentration Final (mM)	Final plasma concentration (mM)	Final tissue concentration (mM)	Tissue concentration: lumen concentration	Tissue concentration: plasma concentration
Control	8	5.55	4.59±0.36	11.19±1.85	15.34±2.35	3.34±0.26	1.37±0.06
Control	5	28	26.12±0.17	11.22±0.14	18.14±2.32	0.69±0.004	1.62±0.04
Phlorizin (5×10 ⁻⁵ M)	8	5.55	5.66±0.04	10.76±0.44	9.68±1.53*	1.71±0.05	0.90±0.02†
Control	4	0	ND	14.95±0.90	8.57±1.48	—	0.57±0.008
Diabetic	6	5.55	4.64±0.06	30.62±2.13	36.13±3.32	7.79±1.96	1.18±0.03

Three small (approximately 30–40 mg wt) and adjacent portions of the freeze-clamped tissue were chipped off and weighed on a torsion balance. The first was ground to a paste in a ceramic mortar containing 6% (w/v) perchloric acid, the extract thawed at 4° C, centrifuged, the supernatant neutralised with 30% KOH and analysed for glucose⁸. The samples of blood, and after centrifugation, plasma, withdrawn from the heart, and the luminal sample, were also deproteinised, and analysed for glucose by the same method. The second tissue sample was ground with 1% (w/v) sodium citrate, to prevent blood clotting, and its absorption measured at 415 nm, since haemoglobin absorbs strongly at this wavelength. A known volume of the whole blood collected from the heart was then added to the tissue extract and the increase in absorption at 415 nm used to calculate the blood volume of the frozen tissue. This averaged 6.6% (in μ l) of the wet weight of the tissue (in mg), which is reasonably close to published figures⁹. The third portion of tissue was dried to constant weight at 110° C to determine the ratio of dry weight to wet weight of the frozen segment. This was invariably less (usually around 0.16) than the true dry weight: wet weight ratio of the tissue (0.215±0.0001 mean±s.e.) determined in separate experiments, on account of the layer of frozen medium sandwiched between the tissue layers, and could be used to calculate the amount of glucose contaminating the tissue from this source. The values for tissue glucose concentration in the above table are therefore corrected for luminal glucose contamination, and for glucose contained in a space accessible to haemoglobin. (The corrected values were about 30% higher than the uncorrected ones.) The intracellular water associated with the tissue was assumed to be the same as the total tissue water, so that the figures for whole wall glucose concentration are probably an underestimate of the true intracellular glucose concentration. Experimental diabetes was induced by the intravenous administration of streptozotocin, 65 mg kg⁻¹, 5 d before the experiment. The ratio of dry weight to wet weight of frozen segments after perfusion from diabetic rats was 0.20±0.0001 and this value was used in the calculation of glucose contamination in these experiments. All results are the mean ± s.e.m. ND, Not determined.

* $P < 0.05$ compared with control.

† $P < 0.001$ compared with control.

sugar from the mucosa during the time taken to sample the tissue.

In the complete absence of glucose in the lumen, and in the presence of phlorizin (Table 1) a substantial amount of glucose was present in the gut wall probably in the mucosa (or possibly the intercellular spaces). If so, one has to postulate a backward movement of glucose from the plasma to the epithelial cells, and strong evidence in support of some form of mediated sugar movement across the basal and lateral surface of the epithelial cells has recently been reported⁸⁻¹⁰. In the case of phlorizin inhibition, one has also to postulate some additional movement of glucose into the epithelial cells, insensitive to the action of this inhibitor.

When experimental diabetes was induced by streptozotocin the plasma glucose rose to 30 mM and provided a sixfold uphill, lumen to plasma concentration gradient. (Table 1.) In spite of this extra load, the tissue responded by raising the intracellular glucose concentration to a level above that of the plasma, to bring about a downhill sugar gradient from tissue to plasma, suggesting that gut glucose concentrations during absorption are influenced more by the concentration of sugar in the plasma rather than that in the lumen.

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In vitro cultivation of *Trypanosoma vivax* isolated from cattle

IN West Africa, *Trypanosoma vivax* is responsible for the majority of trypanosome infections in cattle and other ruminants. It is not readily established in laboratory animals, and research on pathogenic isolates has been hampered by the difficulty in its establishment *in vitro*. Trager¹ reported a culture method for *T. vivax* using tsetse fly tissues but this medium is complex and unsuitable for metabolic studies. There is therefore a need for a medium which will support the *in vitro* growth of *T. vivax* and other species of trypanosomes to give reproducible results. The multiplication of *T. vivax*, using mammalian tissue culture media buffered with 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid (HEPES) is reported here.

Three isolates were used. Bodija 1, Bodija 2, and Vom 36/15. The first two were obtained from blood samples collected from

cattle at the Ibadan slaughterhouse and the third was supplied as a stabilate from the Nigerian Institute for Trypanosomiasis Research (NITR) Vom. The two local isolates were identified as *T. vivax* by morphology and failure to infect rats. Heparinised cattle blood containing approximately 19×10^6 trypanosomes was inoculated intraperitoneally into sheep obtained from the Jos Plateau, a tsetse-free area. The stabilate, Vom 36/15 was injected into a sheep. The parasitaemia, general health and haematology of the sheep were monitored daily.

Trypanosomes for cultivation studies were isolated from whole blood by centrifugation, DEAE cellulose columns², or erythrocyte lysis³ followed by DEAE cellulose columns. The last method was found to be the most efficient, important with the low parasitaemias frequently associated with *T. vivax*.

Isolated trypanosomes were washed twice and resuspended in Hanks saline +5% foetal calf serum, and inoculated into culture media. Some of the media and additives tried are included in Table 1. The number of trypanosomes inoculated into each ml of culture was 0.48×10^6 or 1.0×10^6 . The number in culture at various times after inoculation was determined by dilution in a fixative-staining solution (0.5% toluidine blue, 1% sodium citrate, 10% formalin, in distilled water) and counting in a haemocytometer. When trypanosomes numbered less than 0.03×10^6 ml⁻¹ survival was monitored by evidence with phase microscopy of freely motile trypanosomes. At each sampling, Giemsa stained smears were prepared after centrifugation and resuspending the trypanosomes in serum. NADH diaphorase technique was used to demonstrate mitochondria⁴.

Plasma from infected and uninfected animals was stored at -10°C before incorporation into culture media.

As indicated in Fig. 1, cultures of *T. vivax* (Bodija-1 isolate) were made at parasitaemias of 11, 14, 17, 18, and 22 d after infection of sheep. Table 1 presents the results of the cultures of the 22-d isolate of Bodija 1. Cultured in Medium 199 buffered by HEPES (Medium 199H) with foetal calf serum and homologous plasma (prepared the same day as the culture), and incubated at 25°C , organisms increased fourfold within 24 h. *T. vivax* from the same day isolated in Medium 199H and either γ -globulin free serum or foetal calf serum, increased in numbers 3.8 and 3.1-fold respectively within 24 h. After the initial increase, numbers decreased at 48 h (1.95 to $1.55 \times$

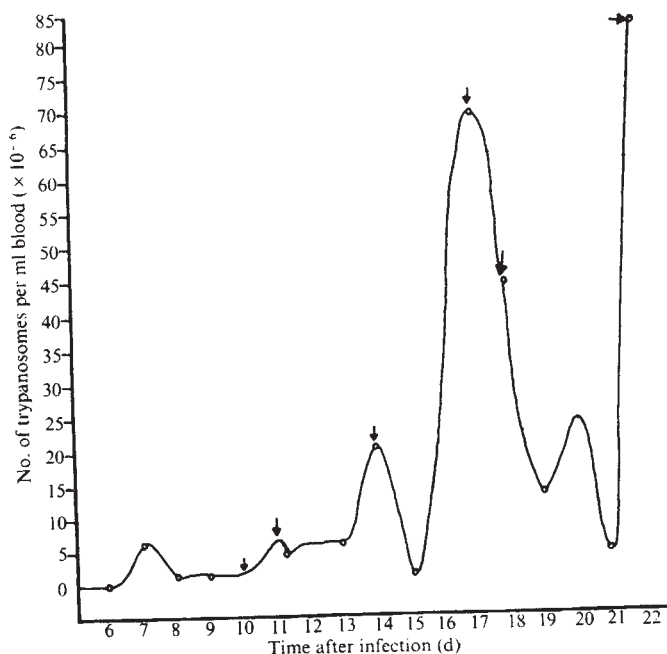


Fig. 1 Parasitaemia of *T. vivax* (Bodija 1) in sheep. Arrows indicate points in parasitaemia where cultures of *T. vivax* were prepared.

Table 1 Growth of *T. vivax* (Bodija 1) at 22 d after infection of a sheep, in various media at 25° C

Medium	No. at 0 h ($\times 10^6$ ml ⁻¹)	No. at 24 h ($\times 10^6$ ml ⁻¹)	Increase ratio (No. at 24 h; No. at 0 h)
199H*†, foetal calf serum†, homologous plasma†	0.48	1.95	4.0
199H, γ -globulin free serum†	0.48	1.86	3.80
199H, foetal calf serum	0.48	1.50	3.10
199H, foetal calf serum, homologous plasma§	0.48	0.66	1.30
Modified Grace's insect medium, haemolymph†			
homologous plasma†	0.48	0.54	1.10
Modified Grace's insect medium, lactalbumin hydrolysate	0.48	0.54	1.10
199H, foetal calf serum erythrocyte lysate§	0.48	0.00	0.00
199H, foetal calf serum, whole blood filtrate*	0.48	0.00	0.00

* Medium 199 with Hanks saline and HEPES buffer.

† Grand Island Biological Co., Grand Island, New York.

‡ Plasma from the infected sheep from which blood was collected for this culture, on day 22 of infection.

§ Plasma from the infected sheep from which blood was collected for this culture, on day 18 of infection.

|| Sigma, St. Louis, Montana.

10^6 ml⁻¹ and 1.5 to 1.05×10^6 ml⁻¹ for example). From day 2 the number of trypanosomes continued to decrease. Throughout the period *in vitro*, however, but more noticeably after subculture every 72 h, there were groups of trypanosomes which appeared to be the result of division (early: rosettes, later: clumps). With Giemsa stained smears, forms which appeared to be dividing were present. Motile trypanosomes were present after 10 d *in vitro* when the cultures were terminated. In Trager's work as well as in long term cultures of primary lines with other species of trypanosomes, initial growth may be followed by a decrease or near disappearance of the line; later resumption of growth occurs from a very few competent organisms. Other media were less successful in supporting the growth of *T. vivax* (Table 1).

The populations of *T. vivax* isolated in Medium 199H and foetal calf serum at 25° C on days 11, 14, 17, and 18 after infection, survived 24, 96, 24, and 72 h, respectively.

The morphology of *T. vivax* *in vitro* during the growth period is similar to that of the forms seen in the whole blood when collected from the host, except that by 4 h *in vitro*, the kinetoplast moved 1–2 μ m anteriorly. The mitochondrion as demonstrated by the NADH diaphorase technique did not seem to be significantly altered during the first 24 h period of growth *in vitro*.

Medium 199H with foetal calf serum and homologous plasma also supported growth at 25° C or 31° C of selected isolates of Bodija 2 and Vom 36/15.

Infectivity studies in ruminants were not carried out in this study as the volume of cultures was too small.

Our data indicate that trypanosomes taken at different times during the course of infection with *T. vivax* behaved differently in identical *in vitro* conditions. *T. vivax* is generally considered monomorphic, although evidence of morphological variation between strains has been reviewed recently⁷. We have observed considerable physiological pleomorphism within strains of *T. vivax*.

The optimum pH for the growth of pathogenic trypanosomes is considered to be 7.4. HEPES buffer⁸ which has an efficient buffering capacity in the pH range 7.4–7.5 (pK 7.31 at 37° C and a pK_a of 0.014) may therefore promote the multiplication of *T. vivax*. The use of homologous plasma seems to enhance growth.

Our method may be useful for the short term cultivation of *T. vivax* for metabolic, biochemical, immunological and pharmacological studies.

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Neuraminidase treatment of adrenal cells increases their response to cholera enterotoxin

A PRIMARY action of cholera enterotoxin is to increase adenylyl cyclase activity in the intestine and the elevated levels of cyclic AMP are thought to be responsible for the water loss and dehydration associated with cholera^{1–4}. Enterotoxin also stimulates a variety of other tissues including cultured adrenal tumour cells^{5,6}. In these instances the actions of the toxin are very similar to those hormones whose effects are mediated through regulation of intracellular cyclic AMP in their target tissues. A common characteristic of the hormonal responses, however, is that cyclic AMP levels are usually raised within a few seconds whereas the response to enterotoxin, in all the tissues tested, is always accompanied by a lag period of at least several minutes. We have shown previously that pretreatment of isolated rat adrenal cells with neuraminidase reduces the response to ACTH indicating that membrane sialic acid may be involved in stimulation of adenylyl cyclase by this hormone^{7,17}. We report here that in sharp contrast to the results obtained with ACTH, the response of adrenal cells to enterotoxin is markedly enhanced by treatment with neuraminidase.

Isolated rat adrenal cells prepared by collagenase-trypsin digestion⁷ respond to enterotoxin with a characteristic lag period of between 30–45 min. Subsequently the rates of both cyclic AMP and corticosterone production are linear for at least 1 h. When the cells are treated with 20 mU ml⁻¹ neuraminidase in conditions shown to liberate membrane sialic acid^{7,17}, the response to enterotoxin is increased. The results in Table 1 indicate that in neuraminidase treated cells, the production of both corticosterone and cyclic AMP is increased at all tested concentrations of enterotoxin; the increase is relatively greater for cyclic AMP than it is for corticosterone and the dose of enterotoxin required to produce maximum stimulation is similar for both corticosterone and cyclic AMP.

Figure 1 shows the time course of corticosterone synthesis by control and neuraminidase-treated cells in the presence of maximally stimulatory levels of enterotoxin. The maxi-

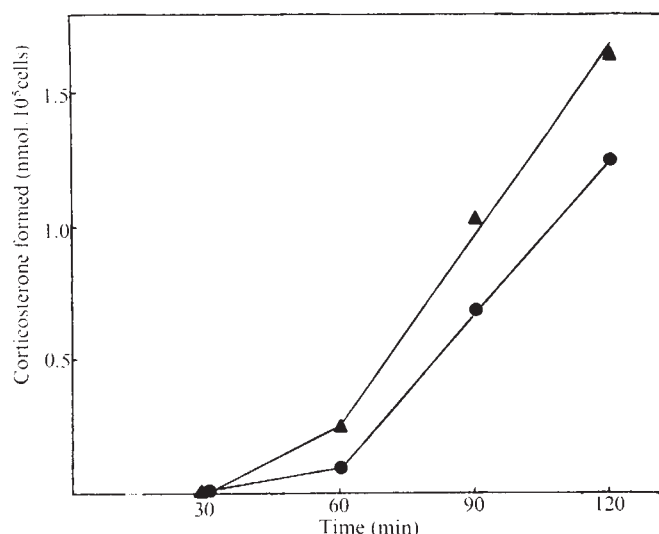


Fig. 1 Time course of corticosterone synthesis by control (●) and neuraminidase-treated (▲) cells in response to enterotoxin. In the absence of enterotoxin, corticosterone production was negligible (<0.05 nmol per 10^5 cells per 2 h) in both groups. Concentration of enterotoxin was $1 \mu\text{g ml}^{-1}$.

imum rates for corticosterone production, attained after 60 min of incubation, are 1.17 and 1.45 nmol per 10^5 cells per h for control and neuraminidase-treated cells respectively. Extrapolation of these curves indicates that the lag period is shortened by about 6–7 min in the neuraminidase-treated cells. Thus, the increased response of neuraminidase-treated cells to enterotoxin may be the result of a combination of two effects: first, the increased rate of synthesis and, second, the shortening of the lag period.

It is not yet clear whether neuraminidase treatment simply 'unmasks' more enterotoxin receptors on the cell membrane or produces a change in the configuration of receptors. Recent studies have indicated that the biological receptor for enterotoxin may be a ganglioside^{8–12}. King and van Heyningen¹⁰ have shown that addition of neuraminidase-resistant monosialosylganglioside (G_{M1}) with enterotoxin can prevent the stimulation of intestinal adenyl cyclase by the latter. Such an effect was not observed with neuraminidase-sensitive di- and trisialosylgangliosides. The treatment of these gangliosides with neuraminidase, however, rendered them capable of abolishing the effect of enterotoxin—presumably by converting them to G_{M1} . Cuatrecasas¹¹ has shown that among all the gangliosides tested, G_{M1} was the most effective in inhibiting the binding of enterotoxin to liver membranes. In the same study it was found that digestion of liver membranes with neuraminidase resulted in a slight enhancement in the ability of the membranes to bind enterotoxin. Thus, it is possible

that increased sensitivity of the neuraminidase-treated cells to enterotoxin in our study was due to the conversion of complex di- and trisialosylgangliosides to monosialosylganglioside in the intact cell membrane. Our studies also provide a plausible explanation for the presence and function of large amounts of neuraminidase found in *Vibrio cholerae*.

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Prostaglandin E_1 action on sinus pacemaker and adenyl cyclase in kitten myocardium

A QUANTUM mechanical analysis of Kier *et al.*^{1,2} indicates that the amine group of the catecholamine isoprenaline may not be essential to produce pharmacological effects mediated through adrenergic β receptors. Based on this and on conformational similarities between the ring, β -hydroxyl group and alkyl areas of *N-n*-propylnoradrenaline and prostaglandin (PG) E_1 , these authors predicted that PGE_1 should be able to interact positively with adrenergic β receptors. Isolated tissues from kitten heart are appropriate systems to investigate effects of drugs which are β -receptor dependent and we tested the predictions of Kier *et al.* by investigating if PGE_1 produced positive inotropic and chronotropic effects and whether these effects are transmitted through the adrenergic β receptors.

It is a general finding that dose response curves of positive inotropic and chronotropic effects of catecholamines are similar when obtained with driven left atrial strips and with

Table 1 Effect of neuraminidase (Nase) treatment of adrenal cells on the formation of cyclic AMP and corticosterone in response to enterotoxin

Toxin (ng ml ⁻¹)	Corticosterone formed nmol per 10^5 cells per 2 h		Cyclic AMP formed pmol per 10^5 cells per 2 h	
	Control cells	Nase treated cells	Control cells	Nase treated cells
0	<0.05	<0.05	0.5	1.4
100	0.54	1.53	2.7	13.7
300	1.28	2.00	4.9	33.9
1,000	1.99	2.63	24.6	48.5
3,000	1.91	2.53	24.4	48.8

Procedures for the preparation of isolated adrenal cells^{7,13} and neuraminidase treatment^{7,17} have been described previously. Cyclic AMP was measured by the protein binding method^{14,15} and corticosterone by sulphuric acid-fluorescence¹⁶.

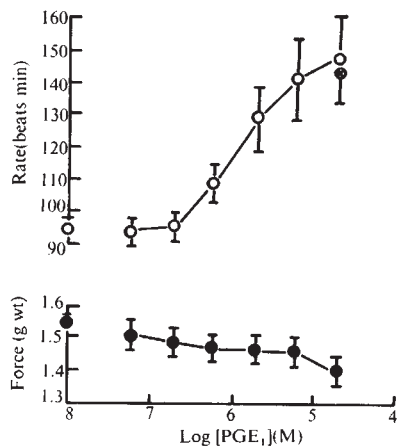


Fig. 1 Nonadrenergic positive chronotropic effect of prostaglandin PGE₁. Tissues were from kittens (weight 350–800 g, either sex), pretreated with reserpine (5 mg kg⁻¹ subcutaneously, 24 h). Cumulative concentration effect curves of PGE₁ on spontaneously beating right atria (○) and on left atrial strips driven at 2 s intervals with barely suprathreshold pulses of 5 ms duration (●). Values are mean ± s.e.m. from four pairs of left and right atria in the same 50 ml organ bath containing gassed (95% O₂, 5% CO₂), warm (32.5°C) modified Krebs solution³. 6×10^{-7} M (±)-propranolol was added for 65 min after 2×10^{-8} M PGE₁ had produced an equilibrium effect on right atria. (±)-Propranolol did not influence the effect of PGE₁ (cross in circle). Open and closed circles on ordinate are values of basal rate and force, respectively, before PGE₁ was administered.

spontaneously beating right atria³. We found that PGE₁ selectively produced positive chronotropic effects but not positive inotropic effects (Fig. 1). Similarly, on six driven papillary muscles (5 s intervals, 32.5°C, from kittens pretreated with reserpine), concentrations from 2×10^{-8} M to 4×10^{-5} M PGE₁ did not modify the strength of isometric contraction or the rates of contractions and relaxation. This dissociation of positive chronotropic effects and lack of inotropic effects of PGE₁ is not characteristic for adrenergic agonists.

To see whether the selective positive chronotropic effect of PGE₁ on right atria was mediated by adrenergic β receptors, β-receptor blocking agents were used. Thus, if PGE₁ is a β agonist its activity should be antagonised by conventional β-blockers and greater concentrations of the prostaglandin should surmount the blockade allowing the estimation of an apparent dissociation equilibrium constant (K_B) for the blocker–receptor interaction. If the blocker and the prostaglandin interact both with the same receptor this K_B should not differ from the K_B estimated from the antagonism of the blocker against a classical β agonist such as isoprenaline. With this in mind, we first added 6×10^{-7} M (±)-propranolol to the atria under the influence of PGE₁. (±)-Propranolol did not modify the positive chronotropic effect of PGE₁ on four right atria (Fig. 1). In two of these atria 1×10^{-7} M (–)-KL 255 was administered 65 min after (±)-propranolol and an additional 30 min incubation of (±)-propranolol plus KL 255 did not influence the positive chronotropic effect of PGE₁. Because the employed concentrations of both (±)-propranolol and (–)-KL 255 were at least 200 times greater than their respective K_B s^{3,4} they produced at least 99.5% β-receptor occupancy. Since the positive chronotropic effect of PGE₁ persisted under these conditions, it must be ruled out that it is mediated through adrenergic β receptors^{1,2,5}.

PGE₁ presumably modifies the ion permeability of the membrane of sinus pacemaker cells in such a way that steeper prepotentials and tachycardia result. If so, it may perhaps be related to an increase of adenylyl cyclase activity in the membrane of pacemaker cells and concomitant increase of intracellular cyclic 3',5'-adenosine monophosphate (cyclic AMP) levels. Based on the previous findings, activation of pacemaker

cell adenylyl cyclase by PGE₁ should be independent of β-receptor action. We therefore explored the possible effect of PGE₁ and isoprenaline on adenylyl cyclase activity in membranes of kitten atria. In such membranes PGE₁ increases adenylyl cyclase activity by 50% (Fig. 2). As expected, this effect of PGE₁ is unaffected by 10^{-7} M (±)-propranolol, indicating that it is not mediated by adrenergic β receptors. To check whether these membranes preserved their β receptors, the action of (–)-isoprenaline was investigated. (–)-Isoprenaline increased adenylyl cyclase activity about three times more than PGE₁. (±)-Propranolol (10^{-7} M) surmountably antagonised the action of (–)-isoprenaline (Fig. 2).

We calculated an apparent K_B of about 3×10^{-9} M for (±)-propranolol from the concentration ratio of concentration-effect curves of (–)-isoprenaline obtained in the absence and the presence of the blocker according to equation $K_B = (\pm)\text{-propranolol}/(\text{concentration ratio} - 1)$. This value of K_B for (±)-propranolol is the same as the K_B between the β blocker and receptors mediating positive inotropic and chronotropic responses of (–)-isoprenaline⁴, indicating that the β receptors are unchanged in cell-free membrane particles⁶.

Concentration effect curves to PGE₁ on both intact right atria—positive chronotropic effect, and on atrial membrane particles—stimulation of adenylyl cyclase, seem to correlate. This correlation does not however necessarily imply functional dependency. If the enhanced adenylyl cyclase activity is a specific step between a hypothetical PGE₁ receptor and a site on the pacemaker cell where permeability changes lead to increased chronotropism, one would expect adenylyl cyclase in membrane particles from the ventricle not to respond to PGE₁. But PGE₁ was found to increase also the adenylyl cyclase activity in membranes from ventricular myocardium (Fig. 3). This effect is only about 2/5 of the maximum increase of activity obtained with (–)-isoprenaline. As in atrial membranes, the effect of PGE₁ is not modified by 1×10^{-7} M (±)-pro-

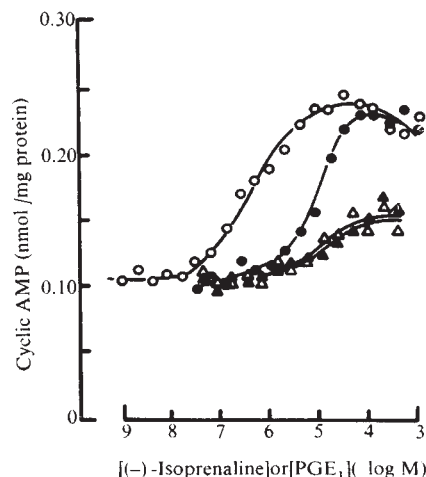


Fig. 2 Nonadrenergic and adrenergic adenylyl cyclase stimulation by PGE₁ and (–)-isoprenaline, respectively. 10,000g membrane particles of atria from kittens pretreated with reserpine were prepared as described elsewhere¹². Particles (78 μg protein) were preincubated in 20 μl of 0.5 mM KHCO₃, 2.5 mM EGTA, 25 mM Tris-HCl, pH 7.5, at 4°C, during 120 min with (solid symbols) or without (open symbols) 10^{-7} M (±)-propranolol; later 10 μl of 1 mM KHCO₃, 0.05 mM EGTA, pH 7.5, with or without (–)-isoprenaline (circles) or PGE₁ (triangles) were added and the mixture further preincubated for 90 min at 4°C. Incubation was initiated by adding 20 μl containing ingredients to give in a final volume of 50 μl: 2.0 mM α-³²P-ATP (1.5×10^6 c.p.m.), 3.5 mM MgCl₂, 1.0 mM cyclic AMP, 20 mM creatine phosphate, 0.2 mg ml⁻¹ creatine kinase and 25 mM Tris-HCl, pH 7.5. The incubation lasted 10 min at 32.5°C; it was stopped with 100 μl of 40 mM unlabelled ATP, 12.5 mM ³H-cyclic AMP (about 10,000 c.p.m.) and 1% sodium dodecyl sulphate followed by immediate boiling for 3.5 min. The ³²P-cyclic AMP formed was determined by the method of Krishna *et al.*¹³.

pranolol and seems therefore not to be mediated by adrenergic β receptors. In contrast, ($\pm$)-propranolol surmountably shifted the concentration-effect curve of (–)-isoprenaline towards greater amine concentrations (Fig. 3) which allowed us to estimate a K_B of about 4×10^{-8} M for ($\pm$)-propranolol. This K_B is the same as the K_B between ($\pm$)-propranolol and β receptors mediating inotropic effects in papillary muscles of kittens, indicating that the β receptors are preserved in ventricular membrane particles⁶. The similarity of K_B s of ($\pm$)-propranolol on membrane particles from atria and ventricles is evidence that the membranes from both tissues have similar β receptors.

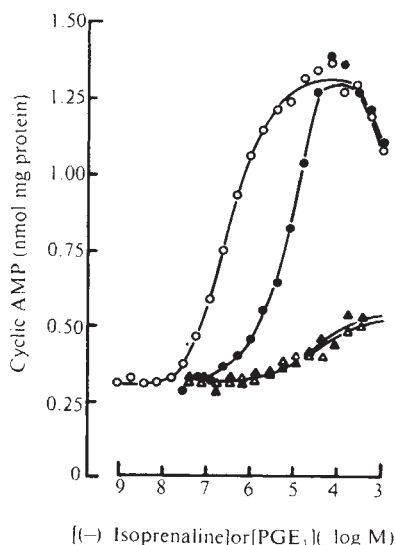


Fig. 3 Comparison of adenylyl cyclase stimulation by PGE_1 (triangles) and by (–)-isoprenaline (circles) on membrane particles of ventricle. For incubation medium and other conditions, see legend to Fig. 2. Solid symbols: with 10^{-7} M ($\pm$)-propranolol; open symbols: without ($\pm$)-propranolol.

Our results support the suggestion that PGE_1 would increase chronotropism through an increase of adenylyl cyclase activity in cardiac membranes. Cyclic AMP has been shown to increase activity of pacemaker cells not only in atria, but also in Purkinje fibres. The increase of heart cyclic AMP⁸, and membrane adenylyl cyclase activity to PGE_1 seems to be of no consequence for inotropism and if PGE_1 promotes chronotropism through stimulation of adenylate cyclase activity, this effect is either restricted to intact pacemaker cells or does not affect mechanisms that lead to increased strength of contraction of myocardium. The fact that PGE_1 stimulates adenylyl cyclase and chronotropism through receptors which are not adrenergic β receptors does not rule out the possibility that catecholamines perhaps catalyse the synthesis of endogenous prostaglandins. This has been suggested for the action of cyclase stimulating hormones in the ovary⁹, the thyroid¹⁰, and stomach muscle¹¹.

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Effect of foetal calf serum and epidermal growth factor on DNA synthesis in explants of chick embryo epidermis

THE epidermal growth factor (EGF) is a peptide hormone which has been isolated from the submaxillary glands of male mice^{1,2}. It enhances proliferation and keratinisation of embryonic and neonatal epidermis *in vivo* and *in vitro*^{3,4}. In spite of these morphologically well documented effects, attempts to demonstrate stimulation of epidermal DNA synthesis have failed⁵. In this communication we report that EGF considerably enhances precursor incorporation into DNA of chick embryo epidermal explants and that the responsiveness of the tissue to the hormone is increased by foetal calf serum.

Explants of epidermis of 9-d-old chick embryos were grown in Eagle's minimal essential medium in the presence of 15% foetal calf serum. By means of pulse-labelling with tritiated thymidine a uniform rate of DNA synthesis was observed over the first 7 d of cultivation. The DNA labelling was decreased if serum was omitted, but EGF strongly stimulated serum-free cultures (Table 1).

In the following experiments the explants were preincubated for one day before addition of the hormone. Fig. 1a shows the time course of the stimulatory effect of EGF on the labelling of DNA in the total absence of serum. Maximal stimulation was observed with $2 \mu\text{g ml}^{-1}$ EGF whereas higher doses led to a decrease (Figs 1 and 2). When the explants were precultivated in the presence of 15% foetal calf serum for one day, the control level of DNA synthesis (without EGF) was found to be approximately twice as high as that achieved without serum (Fig. 1b). Under these conditions an EGF dose as low as $0.02 \mu\text{g ml}^{-1}$ (3×10^{-9} M) was sufficient to induce the maximal response (Fig. 1b). With higher concentrations an inhibition of DNA labelling was observed (Figs 1b and 2).

The response of the tissue to the hormone occurred after a certain lag phase which depended on the EGF dose. In the absence of serum (Fig. 1a) the lag phase was much longer than in serum-containing cultures where it was less than 2 h, the time of the first measurement (Fig. 1b). In order to decide whether the serum factor was identical with EGF, the explants were precultivated with $2 \mu\text{g ml}^{-1}$ EGF instead of serum. One day later a further $2 \mu\text{g ml}^{-1}$ EGF were added and the incubation was continued for 24 h. Marked labelling of the DNA was found (specific radioactivity of $1,880 \pm 143$ c.p.m. μg^{-1} in ten experiments) compared to a control in which the second addition of EGF was omitted (227 ± 42 c.p.m. μg^{-1}). This is in clear contrast to the inhibition of DNA labelling by $2 \mu\text{g ml}^{-1}$ EGF in cultures preincubated with serum instead of EGF (Fig. 1b). The serum effect is thus obviously not due to contamination by EGF.

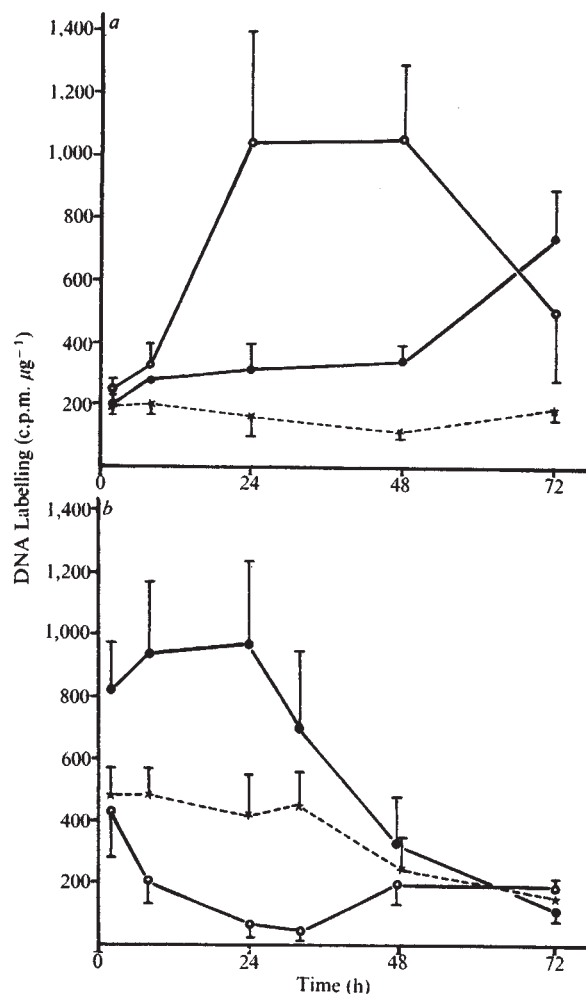


Fig. 1 Effect of epidermal growth factor (EGF) on the pulse-labelling of DNA in chick embryo epidermal explants cultivated in the absence (a) or presence (b) of serum. EGF was prepared from frozen submaxillary glands of male mice according to ref. 2. The explants were dissected from the back-skin of 9-d-old chick embryos (White Leghorn) and epidermal sheets were separated from connective tissue by incubation with 0.1% collagenase (Boehringer Mannheim) in Dulbecco's phosphate-buffered saline (PBS) at 37°C for 25 min⁴. The method used for tissue culture was based on that described by Cohen⁴. Intact sheets of epidermis were layered on to pieces (0.5 × 0.5 cm) of Millipore filter (Solvint, pore size 0.25 µm) with the peridermal side facing upwards. The filters were placed on agar platforms⁴ and incubated in plastic Petri dishes (Falcon, 3.5 cm diameter) with 1 ml Eagle's minimal essential medium (MEM) at 37°C in the presence of 5% CO₂. Each Petri dish contained 3 explants. For DNA labelling three filters with explants were removed from the agar platforms, placed into an Eppendorf reaction vial containing 5 µCi ³H-methyl thymidine (NEN, Boston, USA) in 1 ml medium and incubated at 37°C for 1 h. The medium was then replaced by 1 ml ice-cold 0.4 M perchloric acid and the samples were allowed to stand in an ice-bath for 10 min. After washing with 1 ml ethanol (twice) and 1 ml ether (twice) as well as with 1 ml ice-cold 0.4 M perchloric acid each, each filter was transferred into 0.4 ml 0.3 M NaOH and incubated at 30°C for 90 min. During this procedure the epidermal sheet was solubilised. The filter was removed and the solution was acidified with 0.1 ml 3 M perchloric acid. After 60 min (0–4°C) the precipitate was collected by centrifugation, washed three times with 1 ml 0.4 M perchloric acid and finally dispersed in 0.3–0.4 ml 0.5 M perchloric acid and heated at 95°C for 20 min. Aliquots of the clear supernatant were used for measuring radioactivity and DNA content⁶. The explants were preincubated in MEM (without serum) for 24 h before the addition of EGF. ★ controls (without EGF); ● 0.02 µg ml⁻¹ EGF; ○ 2 µg ml⁻¹ EGF. Each point represents the mean of > 10 determinations (± s. d.).

Table 1 Pulse-labelling of DNA in chick embryo epidermal explants after 24 h cultivation in the absence and in the presence of foetal calf serum (15%) or epidermal growth factor

EGF (µg ml ⁻¹)	Serum	Specific radioactivity of DNA (c.p.m. µg ⁻¹)
—	+	740 ± 240
—	—	348 ± 172
0.2	—	1294 ± 245
2	—	2830 ± 573

For experimental details see Fig. 1. (± s. d., *n* > 10)

These results show that EGF strongly induces DNA synthesis in explants of foetal epidermis and that foetal calf serum increases the tissue's responsiveness to the hormone approximately one hundredfold. Since similar observations could not be made with epidermis of adult animals the stimulatory effect of EGF is apparently restricted to the foetal or embryonic tissue. On the other hand, the healing of wounded corneal epithelium of adult rabbits is accelerated by the factor, although as soon as the regeneration is completed, the effect disappears completely⁹.

We have recently proposed⁷ that the proliferative compartment of foetal epidermis is built up by 'pluripotential' stem cells which are replaced by 'committed' stem cells during adolescence and that epidermal wound healing in adult animals is accompanied by a dedifferentiation of committed, or by an activation of residual 'pluripotential' stem cells (refs 10 and 11 and unpublished data).

Whereas a committed cell population may be considered to be a self-regulated system, in a pluripotential cell population the equilibrium between growth and specific tissue function must necessarily be controlled by exogenous factors. Since EGF has been shown to induce cell proliferation as well as tissue function in interfollicular foetal epidermis^{3,4}, it may be such a factor.

At present it is impossible to tell whether the primary activity of EGF is to induce growth or keratinisation, or whether it does both by controlling tissue homeostasis. If EGF induces keratinisation and the stimulation of growth is due to compensatory proliferation of the remaining stem cells, the inhibitory effect of overdoses of EGF (see also ref. 8) could be explained as the consequence of a rate of keratinisation too high for compensatory proliferation, so that the stem cell pool runs empty.

The ontogeny of epidermis may include several stages of commitment. One of the first might be the induction by specific

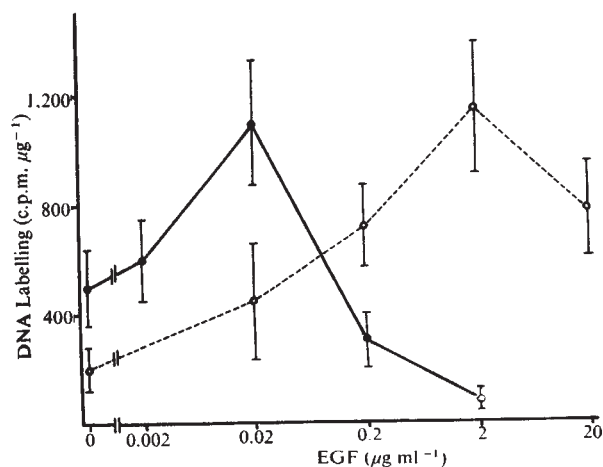


Fig. 2 Dose-response relationship of the effect of EGF on pulse-labelling of DNA in chick embryo epidermal explants. EGF was added after preincubation of the explants for 24 h. The DNA was pulse-labelled after 48 h. The experiment was done either in the absence (○) or in the presence (●) of 15% foetal calf serum. Each point represents the mean of > 10 determinations (± s. d.). For details see Fig. 1.

serum factors of responsiveness to hormones capable of controlling tissue homeostasis, such as EGF. This stage, during which tissue function depends entirely on exogenous stimulation, is replaced by a second stage where the tissue's own regulation, such as the chalone mechanism^{6,11}, is established.

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Heterogeneity of carcinoembryonic antigen and its fractionation by con A affinity chromatography

THE term carcinoembryonic antigen (CEA) was first used by Gold and Freedman to describe a tumour-associated antigen found in the cellular membrane of adenocarcinomas from endodermally derived digestive system epithelia¹. It is conveniently prepared from liver metastases of primary colonic tumours by initial extraction with perchloric acid followed by Sepharose 4B and Sephadex G-200 chromatography^{2,3}. Initial optimism about the specific occurrence of CEA in digestive tract tumours and in the serum of patients with such lesions has not been sustained and CEA is now known to occur in both normal colonic mucosa and other normal tissues⁴ and in the sera of normal individuals, although in relatively low amounts⁵. Elevated levels of CEA have been found in a wide variety of neoplastic diseases and also in non-neoplastic disorders such as gastrointestinal polyps, alcohol cirrhosis, uraemia and inflammatory bowel disease⁶⁻⁸.

It seems possible that the inadequate specificity of CEA radioimmunoassay, as used to detect certain types of human cancer, might result from heterogeneity in the CEA preparation used in the assay and to raise antisera. An associated glycoprotein CEX⁹, separated from some batches of CEA by gel filtration, appears to be immunologically identical to NGP¹⁰ and NCA¹¹ antigens originally reported. Other workers, however, using a variety of techniques^{3,9,12,13}, have demonstrated the heterogeneous nature of CEA which had been separated from CEX. Charge heterogeneity and variation in electrophoretic mobility may reflect variability in sialic acid content although residual heterogeneity of CEA is still seen after neuraminidase treatment. Also, different batches of CEA are known to vary in terms of carbohydrate/protein ratio and their amino acid constitution and it is significant that the most marked variation occurs between preparations from tumours of different tissues¹⁴.

Here we describe the use of con A immobilised on Sepharose 4B (ref. 15) to separate CEA into immunologically active fractions. Of particular relevance to our work are the

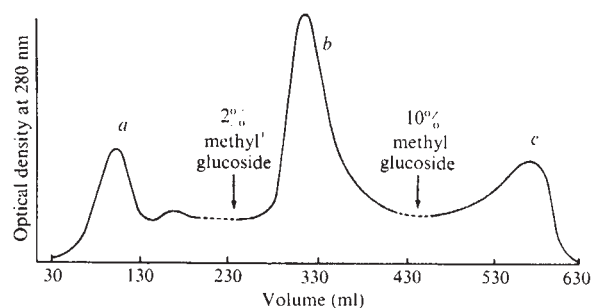


Fig. 1 Con A-Sepharose chromatography of CEA. 30 ml of purified CEA solution² containing 98 mg of CEA was dialysed against 0.1 M sodium acetate buffer (pH 6) containing 1 M NaCl, 10^{-3} M CaCl₂, 10^{-3} M MgCl₂ and 10^{-3} M MnCl₂. The solution was then applied to a column of con A-Sepharose (Pharmacia), bed volume 66 ml and eluted with the above buffer. After the unbound fraction 1 was collected, a 2% solution of methyl glucoside in the above buffer was applied and fraction 2 collected. The third fraction was eluted using 10% methyl glucoside solution. The column was finally washed with 20% methyl mannoside before reuse. The recovery of protein in each fraction, determined by optical density at 280 nm was 10%, 50% and 20% respectively.

results of Chu *et al.*¹⁶ who have shown that CEA can be specifically bound to con A and the binding reversed by another saccharide. CEA used in the present study (M-12) was prepared by the method of Krupay *et al.*² from a large liver metastasis and separated on a column of con A-Sepharose into three fractions as illustrated in Fig. 1. To demonstrate that fraction 1 did not contain some fraction 2 because of overloading of the column, fraction 1 was reappplied to a new column; it was not absorbed and no detectable peak was eluted with 10% methyl glucoside. Each fraction was concentrated to approximately 1 mg ml⁻¹ protein by ultrafiltration using an Amicon PM 10 membrane and tested by double diffusion against various anti-CEA antisera.

The preparation and comparison of the antisera formed the major part of this work. Antisera were prepared in rabbits by injecting 200 µg of a purified batch of CEA³ in complete Freund's adjuvant, intradermally in the flank; a booster was given 40 d later. Antiserum 174, after extensive absorption with pooled normal colonic mucosa extract, normal human plasma and liver extract, produced a reaction of identity in double diffusion with fractions 1, 2 and 3 and an additional reaction with fraction 2 (Fig. 2a). The common antigenic determinant has been designated group A whereas the additional determinant in fraction 2 has been designated group B. Similar results have been obtained with a later CEA preparation (M-31). Anti-NCA (ref. 11) and anti-CEX (ref. 9) antisera on the other hand failed to react with fractions 1 and 2 but gave a strong reaction with fraction 3 identical to the reaction given by NCA previously prepared from a crude CEA extract (Fig. 2b).

These results are in contrast to those obtained with antiserum 224. After absorption, a sharp precipitin line was produced with fraction 1 but no reaction was given with fractions 2 and 3 (Fig. 2c). The determinant with which antiserum 224 reacts is quite distinct from the A group determinant (Fig. 2d) and has been tentatively designated the Y group, although it is not yet clear whether this is a widely occurring CEA component.

Various antisera, such as 'ace' 10, 22, 24 and 36, reacted similarly to antiserum 174 and gave a single line of identity between all three fractions. With the A-group specific line given by the latter antiserum (Figs 3a and 3b) 'ace' 24 antiserum gave an additional line with fraction 3, (cross-reacting with the A-group specific line), which has been shown to be identical with the line given by anti-CEX antiserum and the associated glycoprotein CEX. In addition, 'ace' 36 antiserum produces an additional line against fraction 2 (Fig. 3c) which is identical to the B group-specific line given by antiserum 174. It is not

established whether the B group reported here is the same as the unique CEA determinant recently reported by Tomita *et al.*¹⁷, or alternatively the β -E protein reported by Orjasaeter *et al.*¹⁸ which was shown not to be identical to Gold's original CEA.

To exclude the possibility that our antisera may be reacting strongly with components in normal liver and colon, even after extensive absorption, we have tested the antisera against preparations containing up to 50 mg ml⁻¹ of freeze-dried perchloric acid extract in double diffusion studies. Antiserum 224 and the B-group activity of antiserum 174 failed to produce a precipitin line in any case. Anti-NCA antiserum, however, produced a strong line with the extracts showing the presence of the associated glycoprotein. In addition, strong lines were given between anti-NCA antiserum and extracts (20 mg ml⁻¹) of normal lung and spleen in agreement with the original reports of Mach and Pasztaszeri⁹ and von Kleist *et al.*¹¹. Using antiserum 174 and the monospecific anti-CEA antisera of Todd (which react with the A-group) we have been able to demonstrate the presence of CEA in some normal colon and lung tissue extracts at concentrations of about 50 mg ml⁻¹. (Fig. 3d). The failure to detect both B and Y-group antigens on the other hand may be significant, although pending quantitative estimation by radioimmunoassay, their 'normal' level relative to that of CEA as measured by most anti-CEA antisera, is unknown.

Our results, reported here in preliminary form, show that CEA can be separated into at least two immunologically related fractions in addition to its separation from the well

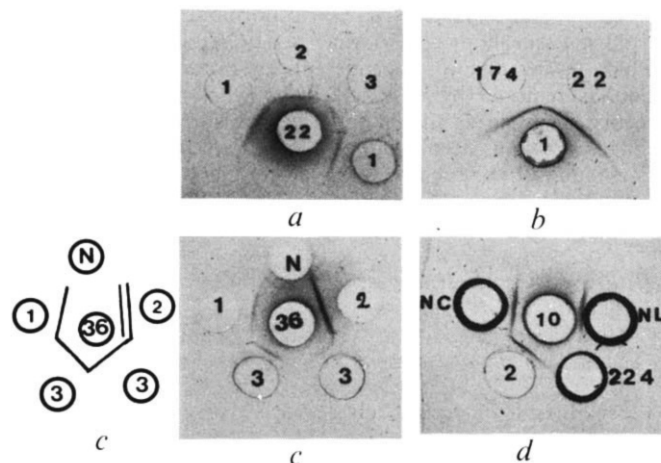


Fig. 3 *a*, Reaction of 'ace' 22 antiserum (centre well) with fractions 1, 2 and 3 showing line of identity. *b*, Reaction of identity between antiserum 174 and 'ace' 22 antiserum against fraction 1. *c*, Reaction showing additional line given by 'ace' 36 antiserum and fraction 2. *d*, Reaction using 'ace' 10 antiserum (centre well) to show the presence of CEA in normal colon (NC) and normal lung (NL); a line of identity with fraction 2 is obtained. Antiserum 224 fails to react.

documented NCA. Also, we have shown that CEA fractions 1 and 2 contain determinants (which may be on separate molecules) which can be distinguished by appropriate antisera. These fractions, however, remain indistinguishable by anti-CEA antisera such as the Todd 'ace' antisera which appear to be reacting with a group common to each of the fractions.

The immunological differentiation between the con A bound and unbound CEA's raises fundamental questions about the chemical nature of CEA. It is necessary to know whether the A and Y and also the A and B groups are situated on different CEA-like molecules and whether further fractionation is possible. It is also important to know how sialic acid is distributed between the CEA fractions and how its removal will affect the binding properties of CEA to con A. In addition to these basic problems, further fractionation of CEA preparations may afford reagents for improving the specificity of the CEA assay and indicate whether circulating CEA shows a similar heterogeneity to preparations isolated from tumour tissue.

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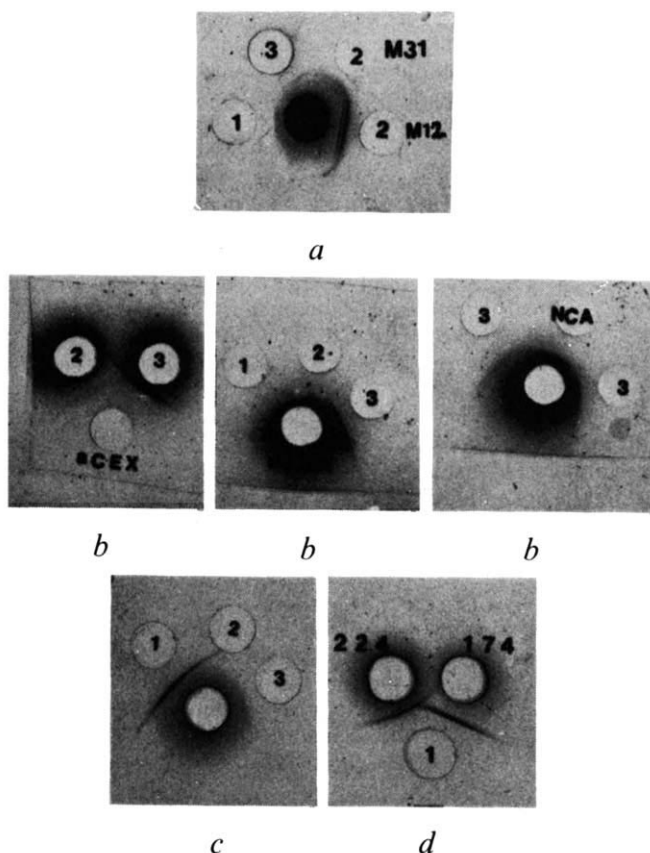


Fig. 2 *a*, Ouchterlony double-diffusion reactions between antiserum 174 (centre well) and fractions 1, 2 and 3 showing line of identity and an additional line produced by fraction 2 from CEA preparations M-12 and M-31. *b*, Reaction of anti-NCA and anti-CEX antisera (bottom wells) with fraction 3 and NCA showing an identical reaction. *c*, Reaction of antiserum 224 (centre well) with fraction 1 and, *d*, reaction showing dissimilar reactions of antisera 224 and 174 against fraction 1.

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Combined hyperthermia and radiation suggest an alternative to heavy particle therapy for reduced oxygen enhancement ratios

THE American Cancer Society estimates that 250,000 people in the United States will die from cancer in 1974 and it has been speculated that 100,000 of them will die because of failure to control the primary tumour site¹. In the report of a 1972 conference on particle accelerators in radiation therapy Bagshaw said: "... perhaps the most critical factor in determining success or failure in the sterilisation of a localised neoplasm with standard megavoltage radiation is the state of oxygenation of the cells being irradiated ..."². Cells which are poorly oxygenated when irradiated are more resistant to conventional X radiation than those which are well oxygenated. Many tumours are hypoxic and thus may be more resistant to radiation than the well oxygenated surrounding normal tissue. Considerable time and effort have been expended during the past decade in attempts to circumvent this problem. Efforts are currently focused on the use of heavy particles. We now have data suggesting that hyperthermia in conjunction with standard megavoltage radiation may present an alternative for circumventing the oxygen problem.

Our interest in the oxygen problem arose from *in vivo* studies of the effects of hyperthermia on the radiation sensitivity of normal and malignant tissues. These studies showed that the radiation sensitivity of C3H mammary tumours in mice was increased by a factor of 4.27 by treatment at 43.0° C, whereas the sensitivity of normal mouse skin was increased by a much smaller factor—2.06 (ref. 3). Since this tumour is considered to be poorly oxygenated, the data suggested a differential effect of hyperthermal treatment on the radiation sensitivity of hypoxic and well oxygenated cells. We present here data that show a selective hyperthermal radiation sensitisation of anoxic cells.

Our biological test system was the spleen-colony bone marrow system of Till and McCulloch⁴. Receptor mice were given a whole body dose of 800 rad which eliminated endogenous haematopoietic cells from the spleen. On the next day, marrow cells were collected from donor mice, counted, subjected to the experimental treatment and then injected into the tail vein of receptor mice. A fraction of these cells lodged in the spleen and grew to form cell aggregates, grossly visible as nodules in the otherwise radiation-depleted spleen. The number of colonies in the spleen 10 d after injection was used as a relative measure of cell survival.

Properly diluted suspensions of bone marrow cells were placed in siliconised test tubes in water baths for 1 h at 37.5°, 42.5° or 43.0° C. This hyperthermal treatment was initiated before irradiation, maintained during irradiation and continued after irradiation, with the heating time equally divided between before and after. This treatment regimen was chosen to allow direct comparison with parallel studies of tumour therapy already in progress. Cells were irradiated with approximately 100 rad min⁻¹ from a conventional 100 KVP X-ray unit.

Hypoxia was produced by causing high purity nitrogen containing 5% carbon dioxide (specified by the manufacturer as having oxygen contamination of less than 2 p.p.m.) to flow over the cell suspensions, which were agitated periodically to assure gas equilibrium. The effluent gas was monitored continuously and the degree of hypoxia was considered adequate when less than 10 p.p.m. of oxygen was detected, although the gas flow was continued for the full hour of thermal treatment. Gas treatment was identical for the oxygenated group, except that air with 5% carbon dioxide replaced the nitrogen-CO₂ mixture. Cells rendered anoxic in this manner and well-oxygenated controls were irradiated, using an appropriate dose range, to determine radiation survival curves at each of the hyperthermal temperatures. Data were obtained for both the anoxic and oxygenated groups during the same period of treatment and at least two independent treatments were carried out at each temperature. Regression analysis was carried out on the survival data to yield the survival curve slopes shown in Table 1.

Table 1 Calculated best fit slopes and associated standard deviations, obtained with hyperthermal treatment

Temperature (°C)	Survival curve slope (10 ² per rad)		OER
	Oxygenated	Anoxic	
37.5	0.94 ± .05	0.38 ± .02	2.47 ± .19
42.5	1.81 ± .18	1.07 ± .06	1.69 ± .19
43.0	2.66 ± .09	1.93 ± .09	1.38 ± .08

OER, oxygen enhancement ratio. This describes the relative radiation sensitivity of the oxygenated and anoxic cells, and was calculated from the ratios of the slopes of the anoxic to oxygenated cells at each temperature.

Although there was a marked increase in the radiation sensitivity of both oxygenated and anoxic cells with increasing temperature of treatment, it was appreciably greater for the latter. Anoxic cells subjected to the hyperthermal treatment at 43.0° C were more sensitive to radiation by a factor of 5.07 (1.93/0.38) and oxygenated cells by a factor of 2.83 (2.66/0.94) than their controls at 37.5° C. This selective radiation sensitisation brings about a reduction in the oxygen enhancement ratio (OER) from a value of 2.47 at 37.5° C to 1.38 at 43.0° C.

To place these data in perspective, Table 2 lists some representative and recently reported OER values from studies with heavy particle beams produced by an accelerator. They range from approximately 1.5 to 2.2. Since the OER of 1.38 which we have obtained through hyperthermal treatment at 43.0° C

Table 2 Recently reported OER values obtained with heavy particle beams under conditions suitable for patient treatment

Particle	OER	References
Pi mesons	1.5–2.2	5
Fast neutrons	1.5–1.8	6
Helium ions	1.55–1.65	7

seems to compare well with those reported from heavy particle studies, our data suggest that the use of hyperthermia as an adjunct to radiation therapy might well be a way to circumvent the oxygen problem. The production of thermal fields necessary for the treatment of patients will require considerable research and engineering. If a technology for producing suitably controlled and localised thermal fields can be evolved, hyperthermia in conjunction with readily available radiation sources (such as cobalt teletherapy units, linear accelerators or betatrons) might provide some of the same advantages as heavy particle therapy.

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Functional identification of three components which mediate tissue-type specific embryonic cell adhesion

VARIOUS hypotheses have been suggested to explain *in vitro* reconstruction of tissues from populations of dispersed embryonic cells¹⁻⁵, all asserting that it is based on selective adhesion. Although selective adhesion has been amply demonstrated⁶⁻⁸, its molecular basis as well as the identity and number of components involved remains to be defined. Supernatant solutions from cell and tissue cultures have been reported to yield a component(s) apparently involved in the enhancement of cell aggregation⁹⁻¹¹. In addition a component(s) in the supernatant binds at the cell surface and is specific to the cell type from which the supernatant is prepared¹¹. These results suggested the existence of specific receptors at the cell surface that are complementary to the aggregation promoting molecules (APM). We have now found that the binding factor participates directly in adhesion and that another component must be present for adhesion to be complete.

Collection of ³H-glucosamine-labelled aggregation-promoting supernatant solutions (APMs; retina aggregation promoting material (RAPM) and cerebral lobe aggregation promoting material (CLAPM, called BAPM in ref. 11)), preparation of single cells and binding of APMs to the cell surface was achieved as before¹¹. After binding (20×10^6 cells per ml of RAPM and 5×10^6 cells per ml of CLAPM), cells with APM or control cells were pelleted and resuspended in 1% glutaraldehyde in phosphate-buffered saline at pH 7.2 for 30 min at room temperature. The fixed dead cells were washed in the cold with six changes of Tyrode solution for 48 h before use.

After glutaraldehyde fixation, neither control cells nor those fixed with APM adhered to one another, even when brought into contact on a gyratory shaker. Cells fixed after binding of ³H-APM retained approximately 50% of bound radioactivity and lost no more label during either washing or a subsequent 24 h incubation at 37° C. Thus fixation bound the APM permanently to the cells and caused no generalised stickiness which could complicate later experiments.

When added to freshly dispersed live cells of the same type, control fixed cells (no bound APM) had no effect on aggregation while APM-fixed cells enhanced the size of the aggregates

formed (Figs 1 and 2). A minimum of one APM-fixed cell for each nine freshly dispersed live cells was required for maximal enhancement of retina cell aggregation while for cerebral cells the ratio was one in five. When, using these ratios of cells, the assay was carried out in the presence of $5 \mu\text{g ml}^{-1}$ cycloheximide (a concentration that inhibits 98% of ¹⁴C-leucine incorporation within 10 min and 100% of aggregation of embryonic cells, our unpublished observations), fixed cells did not mediate any aggregation. This suggests that fixed cells alone do not mediate adhesion of live cells but that live cells must contribute some essential component(s), which was synthesised during our experiment. If this component were newly synthesised APM, addition of APM alone to APM-fixed cells should have caused adhesion or agglutination. This was not the case, further suggesting that the component(s) contributed by the live cells differs from the active factors in APM preparations.

Table 1 shows that fewer single cells remained in homologous combinations of live cells after 24 h with APM fixed cells than with control fixed cells. Thus APM-fixed cells interact with live cells in enhancing aggregation. We have evidence (manuscript in preparation) that APM is bound to the cell surface through the carbohydrate moiety of a macromolecule containing both carbohydrate and protein; for exhaustive pronase digestion does not affect binding of ³H-glucosamine-labelled APM. To further characterise the role of the protein moiety of the surface-bound APM, APM preparation was digested with pronase before binding and fixation. As with whole APM, bound radioactivity was not removed during washing or culture. After washing, fixed cells were combined with live cells at ratios of 1:1 to 1:10. No live cells were promoted to aggregate (Fig. 1d). Results were similar when 10-d embryonic chick neural retina (Fig. 1) and 10-d cerebral lobe cells (Fig. 2) were combined in the appropriate combinations with fixed cells.

Taken together these results suggest that the live cells contribute a component(s) to the adhesive mechanism which differs from APM, and that its activity is mediated through the protein portion of the APM molecule. We have obtained further support

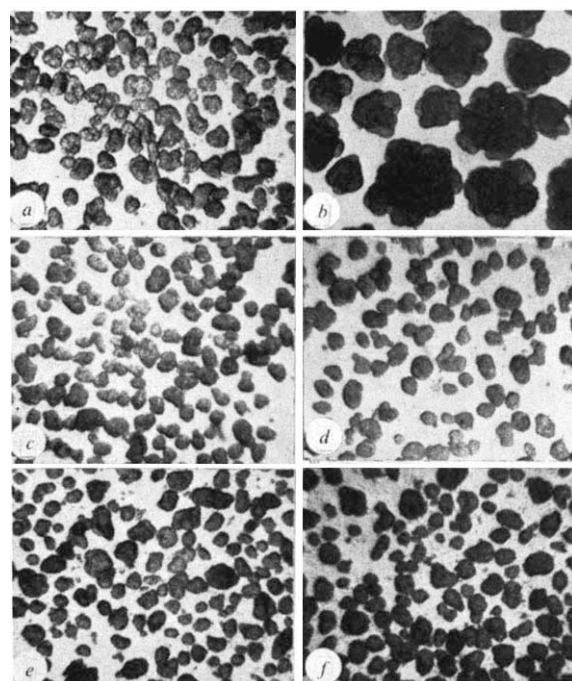


Fig. 1 Aggregation of 18×10^6 10-d retina cells in 3 ml of Eagle's basal medium under an atmosphere of 10% CO_2 in air on a gyratory shaker for 24 h. There were 2×10^6 fixed cells in each case. a, No fixed cells; b, RAPM-fixed retina cells; c, control fixed retina cells; d, pronase-digested-RAPM-fixed retina cells; e, CLAPM-fixed cerebral cells; f, control fixed cerebral cells. ($\times 15$).

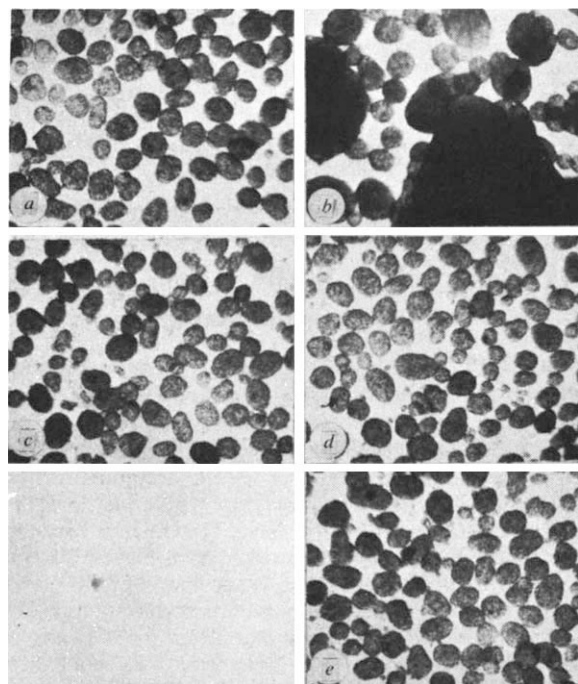


Fig. 2 Aggregation of 4×10^6 cerebral cells as in Fig. 1. *b-e*, 1×10^6 fixed cells; *a*, no fixed cells; *b*, CLAPM-fixed cerebral cells; *c*, control fixed cerebral cells; *d*, RAPM-fixed retina cells; *e*, control fixed retina cells. ($\times 15$).

for the existence of this third component (we consider APM and its receptor to be separate components). If cells are synthesising a third type of molecule which mediates aggregation, monolayer cultures should synthesise and release it into the medium. APM-fixed cells in rotation culture on top of monolayers should thus be agglutinated in the medium above the monolayer. (Agglutinated here distinguishes loose clusters of dead cells from the formation of tissue-like aggregates of live cells. Fixed dead cells, although their adhesive mechanism may be reconstituted, would be unable to undergo further histotypic organisation.)

Monolayer cultures were prepared in Eagle's basal medium without serum; after incubation for 24 h at 37°C they were washed twice with warm serum-free culture medium, and control fixed (no APM) or APM-fixed cells were added. Incubation continued at 37°C on a gyratory shaker for 24 h (Fig. 3). APM-fixed cells were agglutinated maximally (Fig. 3*b* and *f*) while controls showed little or no agglutination (Fig. 3*c* and *g*). These results are not due to monolayer cells which were loosened from the substrate as few if any were released (Fig. 3*a* and *e*). Thus our third component can be active in a form unattached to live cells.

To test the specificity of the component(s) we prepared heterologous combinations between APM-fixed cells and live cells. Aggregation assays revealed little or no ability of APM-fixed cells to enhance aggregation even at high ratios of fixed to live cells (Fig. 1*e* and *f*, Fig. 2*d* and *e*). Rotation culture of APM-fixed cells on top of heterologous monolayers gave essentially the same results (Fig. 3*d* and *h*). Agglutination was always greatest with homologous combinations but some occurred with heterologous combinations. Counts of single cells remaining after 24 h also reflect the specificity of the proposed third component (Table 1). This is evident since in heterologous combinations the number of single cells remaining is the same for APM-fixed and control cultures, while in homologous combinations more single cells remain in control cultures than in APM-fixed cultures.

Various models are consistent with our results. For example, the third component may modify APM in some way to make it capable of forming a functional adhesion, or APM may modify

Table 1 Effect of APM-fixed cells on the number of single cells remaining in suspension after 24 h of rotation culture

Live cells	Fixed cells	Single cells remaining after 24 h	Control fixed-APM-fixed
	RAPM-retina 2×10^6	$2.2 \times 10^6 \pm 0.3 \times 10^6$	
	Control retina 2×10^6	$3.4 \times 10^6 \pm 0.3 \times 10^6$	1.2×10^6
Retina cells 18×10^6	CLAPM-cerebral 2×10^6	$1.4 \times 10^6 \pm 0.2 \times 10^6$	
	Control cerebral 2×10^6	$1.3 \times 10^6 \pm 0.3 \times 10^6$	-0.1×10^6
	CLAPM-cerebral 1×10^6	$0.9 \times 10^6 \pm 0.1 \times 10^6$	0.5×10^6
	Control cerebral 1×10^6	$1.4 \times 10^6 \pm 0.1 \times 10^6$	
Cerebral cells 4×10^6	RAMP-retina 2×10^6	$2.8 \times 10^6 \pm 0.1 \times 10^6$	
	Control retina 2×10^6	$2.8 \times 10^6 \pm 0.3 \times 10^6$	0

Single cells were counted on a Coulter model B particle size analyser at the appropriate settings for each cell type. These results are each the average of six experiments.

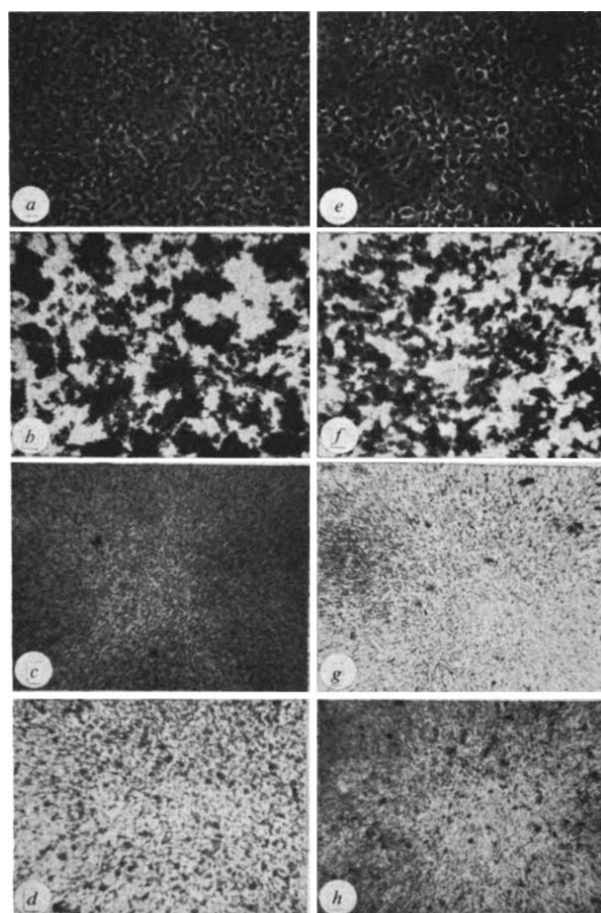


Fig. 3 Agglutination of fixed cells (2×10^6 retina cells or 1×10^6 cerebral cells) in rotation culture on monolayer cultures of retina cells (*a-d*) or cerebral cells (*e-h*) for 24 h. *a, e*, No fixed cells; *b, f*, homologous APM-fixed cells; *c, g*, homologous control fixed cells; *d, h*, heterologous APM-fixed cells. (*a, e*, $\times 96$, focused at plane of monolayer; *b-d* and *f-h*, $\times 15$, focused above monolayer.)

the cell surface, thus allowing third component alone to form a functional adhesion. Or the third component may act as a ligand (Fig. 4). According to this model APM interacts specifically through its carbohydrate moiety with a cell surface receptor. The third component(s) interacts with the protein moiety of APM, again specifically, to effect a bridge between the cells. In this model the third component appears to be functionally

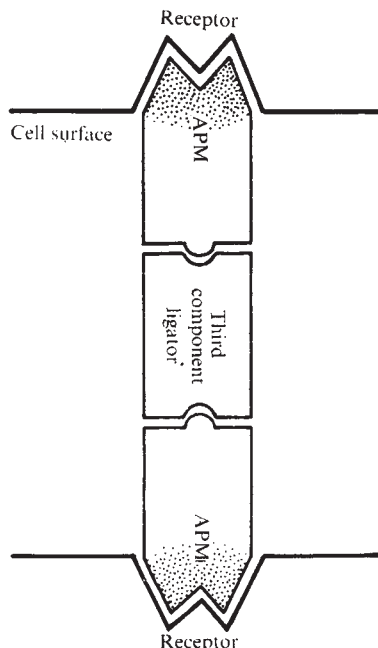


Fig. 4 Model showing the role of each of the proposed components in forming a complete specific adhesive bond. The stippled area is carbohydrate.

analogous to the sponge aggregation factor^{12,13}. The aggregation-promoting factor, or APM as we term it, seems to be functionally analogous to the base plate molecule¹⁴, in that APM and baseplate mediate the effect of the ligator molecule. Although no component analogous to our specific receptor has been identified in the sponge system, Weinbaum and Burger¹⁴ found that once baseplate is removed from cells by hypotonic shock it can be added back to the cells, effecting a functional union.

The existence of the third component(s) may explain the necessity for biosynthetic activity even when APM is supplied to the cells⁹. Immediately after trypsinisation both APM and the third component are removed from the cell surface, for control fixed cells can neither enhance the aggregation of live cells nor be agglutinated when placed on a monolayer of live cells. Aggregation thus requires the replacement of both APM and third component. When APM is supplied a complete adhesive bond is still not formed; the third component is now the limiting factor. Our preliminary observations suggest that this third component is extremely labile. This observation may bear on the need for continued biosynthetic activity during aggregation. If this component is continuously destroyed during aggregation then its continued synthesis would be necessary.

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TRH potentiates behavioural changes following increased brain 5-hydroxytryptamine accumulation in rats

WHEN tryptophan is administered to rats pretreated with a monoamine oxidase (MAO) inhibitor there results a characteristic syndrome of hyperactivity. This syndrome seems to be due to the increased synthesis and accumulation of the putative neurotransmitter 5-hydroxytryptamine (5-HT) and its 'spill-over' into functional activity which is presumably dependent on the stimulation of postsynaptic 5-HT receptors¹. A qualitatively similar syndrome is produced by the administration of 5-methoxyN,N-dimethyltryptamine which, it has been suggested, directly stimulates 5-HT postsynaptic receptors². This hyperactivity syndrome has been used to investigate the functional organisation of brain 5-hydroxytryptamine, the action of drugs such as chlorpromazine³, reserpine¹ and lithium³ and to investigate the role of brain catecholamines⁴ in the mediation of brain 5-HT function.

Recently a report⁵ has suggested that thyrotrophin releasing hormone (TRH) produced a rapid, though transient, improvement in depression when given intravenously. Because the combination of tryptophan plus a monoamine oxidase inhibitor has antidepressant properties⁶ and because reserpine⁷ and lithium^{8,9} also affect mood in man, we investigated the effect of TRH on the hyperactivity syndrome.

Rats were pretreated intraperitoneally with 2 mg kg⁻¹ TRH (Calbiochem, London) 3 h before the administration of 20 mg kg⁻¹ tranylcypromine (Tep), 30 min after which L-tryptophan was given. As shown in Fig. 1a, TRH caused an increase in the degree of hyperactivity¹. There was no potentiation with a TRH dose of 0.25 mg kg⁻¹, perceptible potentiation with 0.5 mg kg⁻¹, a maximum potentiation at 1 mg kg⁻¹ and 2 mg kg⁻¹ but no potentiation when the dose was increased to 10 mg kg⁻¹. With a dose of 1 mg kg⁻¹ it was observed that there was some potentiation of hyperactivity when TRH was given 1 h before Tep and tryptophan, a marked response after 3 h and 6 h, but no effect at 24 h. A 1 mg kg⁻¹ dose of an equimolar solution of proline, histidine and glutamic acid injected instead of TRH (pyroglutamyl histidyl prolylamide) failed to produce potentiation, showing that the constituent amino acids in combination were ineffective. No hyperactivity occurred after TRH pretreatment when either Tep or tryptophan alone were administered.

Pretreatment with either thyroid stimulating hormone (TSH, 1 mg kg⁻¹) or L-thyroxine (1 mg kg⁻¹) (both from Sigma, London) 3 h before the Tep, with L-tryptophan after a further 30 min failed to produce potentiation of the hyperactivity suggesting that TRH was not producing its effect by its action on pituitary secretion of TSH by a TSH-mediated effect on thyroid function. This was confirmed by the observation that TRH still potentiated the hyperactivity in rats hypophysectomised 48 h previously.

TRH alone had no effect on brain tryptophan or 5-HT concentrations nor did it affect brain tryptophan concentrations, 5-HT concentrations or the rate of brain 5-HT accumulation after the administration of Tep and L-tryptophan. The effect of TRH on the hyperactivity produced by 5-methoxy N,N-dimethyltryptamine (5-MeODMT) was therefore examined to see whether TRH was effecting the postsynaptic response to activation of 5-HT receptor sites.

Rats were pretreated with TRH (1 mg kg⁻¹) followed 3 h

later by Tcp (20 mg kg⁻¹) with 5-MeODMT (2 mg kg⁻¹) after a further 30 min. TRH pretreatment consistently increased the degree of hyperactivity which was prolonged following 5-MeODMT (Fig. 1b). This suggested that TRH was altering either 5-HT receptor sensitivity or the postsynaptic responses to increased 5-HT release. When rats were pretreated with TRH (10 mg kg⁻¹) and subsequently challenged with Tcp and 5-MeODMT as above no potentiation was seen. This is consistent with the observation that this higher dose of TRH does not potentiate the hyperactivity following Tcp and L-tryptophan.

Previous experiments have shown the importance of brain dopamine in the production of the hyperactivity following increased 5HT synthesis or 5-MeODMT. The results were tentatively interpreted as indicating that there may be a group of dopaminergic neurones lying between the 5-HT neurones initiating the response and those mechanisms responsible for the expression of the syndrome⁴. Furthermore, Plotnikoff *et al.*¹⁰ showed that TRH potentiates the hyperactivity resulting from administration of a MAO inhibitor and L-dopa; a syndrome which is nevertheless qualitatively different from that produced by increasing 5-HT synthesis. It was possible therefore that TRH might affect the 5-HT induced hyperactivity by an action on the postsynaptic dopaminergic system postulated. We therefore attempted to confirm the visual scoring of Plotnikoff *et al.*¹⁰ by activity meter measurements.

Pretreatment with TRH 3 h before the Tcp and L-dopa potentiated the hyperactivity, both at a TRH dose of 1 mg kg⁻¹ and 0.25 mg kg⁻¹ (Fig. 2). This lower dose had been ineffective in enhancing Tcp/L-tryptophan hyperactivity. This dose, however, did potentiate hyperactivity following Tcp and DL-5-hydroxytryptophan (50 mg kg⁻¹). We are unable, at present, to suggest reasons for these dose-response differences.

There was no alteration in whole brain concentrations of dopamine and noradrenaline 3 h after TRH administration or after a further 90 min when Tcp and L-tryptophan had also been administered.

There have now been several reports of alterations in behavioural responses to released central neurotransmitters

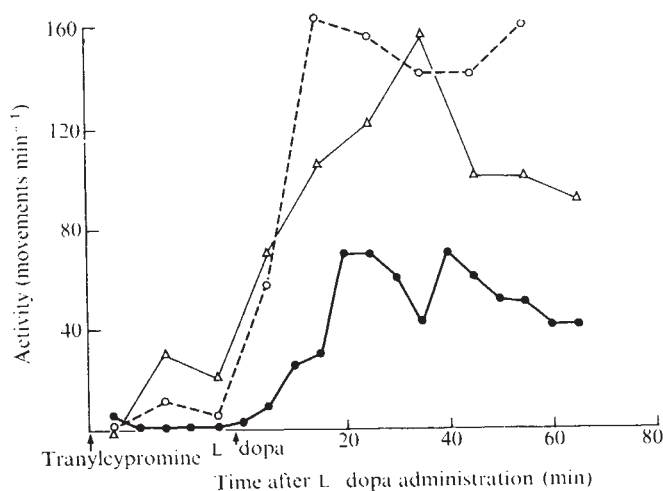


Fig. 1 Hyperactivity response to, *a*, L-tryptophan and, *b*, 5-methoxyN,N-dimethyltryptamine (5-MeODMT) following TRH. Male Wistar rats (150–200 g) were used in all experiments and were pretreated with either TRH 2 mg kg⁻¹ (○) or saline (●). 3 h later both groups were given tranylcypromine (20 mg kg⁻¹) with L-tryptophan (50 mg kg⁻¹) or 5-MeODMT (2 mg kg⁻¹) 30 min later. Activity was measured in groups of three rats on Animex activity meters (sensitivity and tuning setting: 30 μ A) with total movements recorded each minute. Results are plotted against time after tranylcypromine administration and show typical results of experiments performed at least three times.

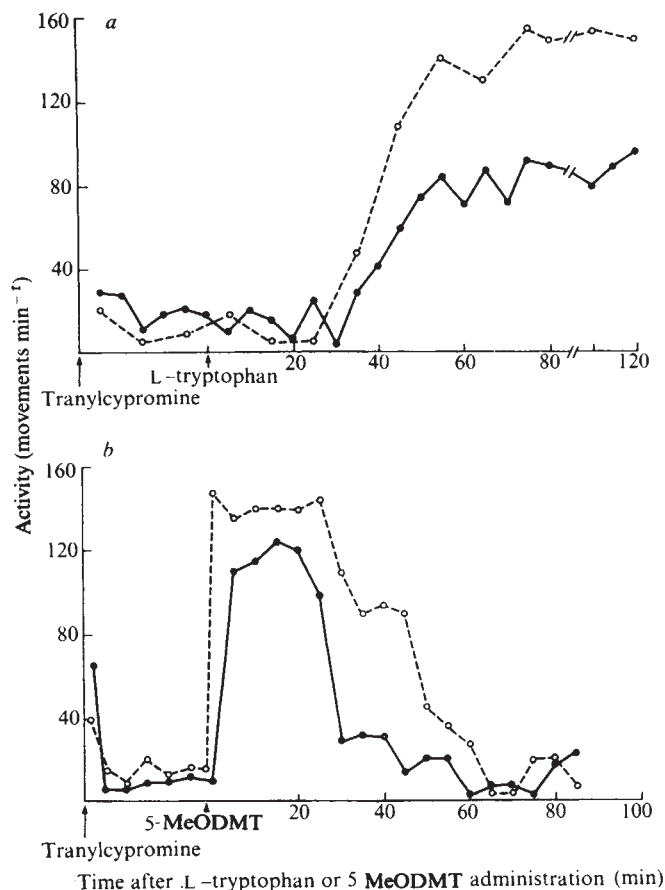


Fig. 2 Hyperactivity response to L-dopa following TRH. Rats pretreated with 1 mg kg⁻¹ TRH (○), 0.25 mg kg⁻¹ (△) or saline (●). 3 h later all groups were injected with tranylcypromine (20 mg kg⁻¹) with L-dopa (50 mg kg⁻¹) administered 30 min later.

following administration of hypothalamic hormones. Plotnikoff *et al.*¹⁰ reported that TRH enhanced the hyperactivity following MAO inhibition and L-dopa and the same group¹² found similar enhancement when rats were pretreated with melanocyte stimulating hormone inhibitory factor (MIF) another hypothalamic hormone with suggested antidepressant properties¹³. MIF also elicits stereotyped behaviour in cats indistinguishable from that following L-dopa or amphetamine¹⁴. Since amphetamine affects dopaminergic systems in the brain¹⁵, MIF may thus be altering the function of dopaminergic neurones. This may also be the case with TRH since we have found that the hyperactivity resulting from injection of 4-methoxyamphetamine (10 mg kg⁻¹) 30 min after Tcp (20 mg kg⁻¹) was also potentiated by pretreatment 3 h previously with TRH (1 mg kg⁻¹).

Another possibility examined was that TRH might increase the overall sensitivity of neurones to excitatory stimuli. We therefore pretreated groups of mice with TRH (1 mg kg⁻¹) or saline and 3 h later pentylenetetrazol (Leptazol; 70 mg kg⁻¹) was given intraperitoneally. In the animals which had received TRH, convulsions occurred with a shorter latent period than those receiving saline (Fig. 3). At present, however, it is not possible to exclude the possibility that this effect of TRH is mediated by some change in the function of brain monoamines.

Recently it has been reported¹⁶ that TRH increased the turnover of cerebral noradrenaline. No change in the brain content of 5-HT, noradrenaline, dopamine, homovanillic acid or 5-hydroxyindoleacetic acid was found in this study. The TRH dose used, however (10 mg kg⁻¹) caused no potentiation of the hyperactivity following Tcp and either L-tryptophan or

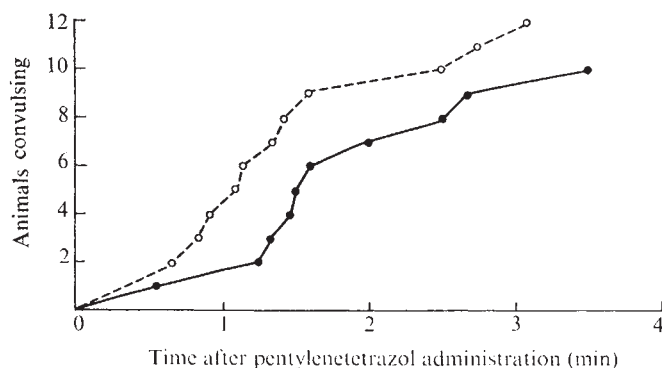


Fig. 3 Time taken for a group of 24 mice given either 1 mg kg⁻¹ TRH (○) or saline (●) to convulse following injection of pentylenetetrazol (70 mg kg⁻¹) 3 h later. All 12 animals injected with TRH convulsed whereas only 10 of the 12 saline injected animals convulsed. Therefore nonparametric statistics (Mann-Whitney test¹¹) were used for analysis. TRH-treated mice convulsed with a significantly shorter latent period than control mice ($T < 0.01$).

5 MeODMT and we have so far been unable to definitely incriminate noradrenaline in the hyperactivity response to increased 5-HT accumulation⁴.

These results show that TRH, in a curiously restricted dose range, causes potentiation of the hyperactivity response to stimulation of 5-HT receptors in the brain. Whether this is due to some effect of TRH to alter neuronal sensitivity generally, as might be inferred from the potentiation of the various effects of L-dopa and Tcp, 4-methoxyamphetamine and pentylenetetrazol—or more specifically through an effect on a system common to all these stimuli—cannot at present be concluded.

These experiments and those of others show conclusively, however, that TRH has actions on brain function quite independent from its effects on TSH and therefore thyroid function.

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γ-Aminobutyric acid synthesised in the olfactory nerve

We have found the candidate neurotransmitter γ-aminobutyric acid (GABA) and its biosynthetic enzyme in the olfactory nerve of two fish. In the light of these findings current concepts of GABA as an exclusively inhibitory transmitter may have to be reconsidered. The olfactory nerve is a component of the rhinencephalon, a phylogenetically primitive part of the central nervous system (CNS). In addition to its receptive sensory function, the olfactory nerve conducts impulses. Fish have either short olfactory nerves and long olfactory tracts, as do most vertebrates, or long nerves and short tracts, as found in the gar fish (*Lepisosteus osseus*) and the pike (*Esox estor*)¹. The olfactory cilia and nerve cell body are in the olfactory mucosa, from where the nerve action potentials are propagated to the olfactory bulb, 3 cm (pike) to 15 cm (gar) away, depending on the size of the animal. The primary synapse occurs within the olfactory bulb with the mitral cells. The olfactory nerve in the gar and the pike contains no motor nor other known sensory fibres^{2,3}. Furthermore, it is neurally homogeneous, sensory, unmyelinated and is of a reasonable size for biochemical study (50–150 mg per nerve per fish).

Fish were caught by net in the Mississippi River, stored and transported to the laboratory in small pools and used as needed. The general methodology of Hildebrand *et al.*⁴ was used to test for the biosynthesis of acetylcholine, dopamine, noradrenaline, adrenaline, serotonin and γ-aminobutyric acid. Gar nerve explants or brain slices (20 mg) were incubated in 500 μl gar Ringer (112 mM NaCl, 3.5 mM KCl, 3 mM CaCl₂, 24 mM glucose and 4 mM N Tris (hydroxymethyl)methyl-2-aminoethane sulphonic acid) containing 5 μCi of either ¹⁴C-L-tyrosine, ¹⁴C-L-tryptophan, ¹⁴C-choline or ¹⁴C-L-glutamate (each 50 Ci mol⁻¹). After 3 h at ambient temperature (23° C), tissues were blotted, homogenised (glass-to-glass) in 120 μl 0.5 N formic acid containing carrier and subjected to paper electrophoresis to resolve the possible neurotransmitter from the labelled precursor. There was no detectable incorporation of tyrosine into dopamine, noradrenaline or adrenaline, of tryptophan into serotonin, nor of choline into acetylcholine in the olfactory nerve. On the other hand, there was substantial incorporation of L-glutamate into a substance which comigrated with GABA on paper electrophoresis. Labelled dopamine, noradrenaline, serotonin, acetylcholine and GABA were formed in the brain slices from their precursors. The demonstration of GABA formation in gar olfactory nerve prompted an investigation of its metabolism *in vitro*.

GABA is an established inhibitory neurotransmitter acting at the crustacean neuromuscular junction and a probable neurotransmitter in the vertebrate CNS^{5,6}. The biosynthesis of GABA from L-glutamate is catalysed by glutamate decarboxylase (EC 4.1.1.15) and the concentration of GABA parallels that of its biosynthetic enzyme⁵. We found glutamate decarboxylase activity in homogenates of olfactory nerve (Table 1) at 25% of the level found in the brain, although the specific activity in the pike (per unit weight of tissue) was about half that in the gar. Enzyme activity in other tissues was very low or not detected (<5 nmol min⁻¹ g⁻¹). Glutamate decarboxylase activity was also present in the gut homogenate from the pike, but not the gar. Roberts *et al.* have reported that a second glutamate decarboxylase (GAD II) is present in non-nervous tissue^{8,9}. The activity might also represent enzyme present in bacterial flora although we removed the flora from the tissue by extensive rinsing before homogenisation.

When we measured GABA as described before¹⁰ (Table 2) we found that nerves contained 65% and 75% of the amount found in the brain of the pike and gar, respectively. None was found in several other tissues (Table 2) except for the gut of the pike. The GABA content thus parallels that of glutamate decarboxylase (Table 1). The level of GABA in the fish brains was about 50–60% that of rat brain¹¹. It was similar to that found in the mammalian CNS¹² but not that in the inhibitory

Table 1 Glutamate decarboxylase activity

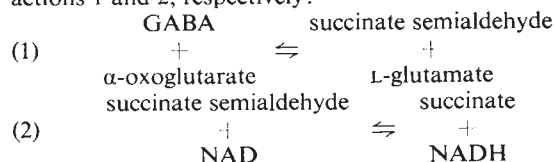
Tissue	Gar (nmol min ⁻¹ g ⁻¹)	Pike (nmol min ⁻¹ g ⁻¹)
Olfactory nerve	80.1	40.9
Brain (whole)	320	162
Gill	< 5	< 5
Visceral fat	< 5	< 5
Gut	< 5	17.4
Skeletal muscle	< 5	NS

A 1:10 tissue homogenate was made in 5 mM potassium phosphate (pH 7.4)–0.1 mM EDTA (buffer *A*). Then 10 μ l of extract was added to 10 μ l of solution so that the final incubation mixture contained 2.0 mM ¹⁴C-L-glutamate (20 Ci mol⁻¹), 0.1 mM pyridoxal phosphate and 50 mM potassium phosphate (pH 7.4). After incubation for 5 min at 37° C, 10 μ l of 0.5 N formic acid–25 mM GABA was added to stop the reaction. Then 10 μ l aliquots were taken for paper electrophoresis⁷. After drying at 100° C for 15 min, the GABA zones were developed with ninhydrin spray and cut out for radioactivity measurement by liquid scintillation counting⁷.

NS, not studied.

motor neurones of the peripheral nervous system of the lobster (about 100 μ mol g⁻¹) (ref. 13).

Two mitochondrial enzymes are required for GABA catabolism. The first, GABA- α -oxoglutarate transaminase, and the second, succinate semialdehyde dehydrogenase, catalyse reactions 1 and 2, respectively:



These enzymes are ubiquitous in tissue, although they are most active in nervous tissue¹⁴. The activity of GABA transaminase was highest in brain and olfactory nerve, but present in all tissues assayed (Table 2).

Glutamate decarboxylase is often considered a marker for neurones for which GABA is the neurotransmitter⁵, and so we characterised it in the olfactory nerve. In gar and pike olfactory nerve homogenates, the time course of GABA biosynthesis was linear for more than 20 min at 37° C (see legend to Table 1). In each case, activity was proportional to protein concentration up to 5.5 mg protein per ml of incubation medium (higher protein concentrations were not tested).

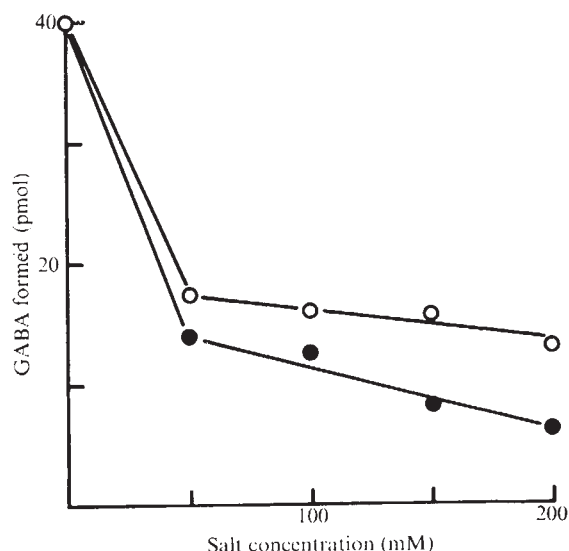


Fig. 1 Inhibition of pike olfactory nerve glutamate decarboxylase by salt. Assays were carried out as described in Table 3 using the specified concentration of KCl (●) or NaCl (○).

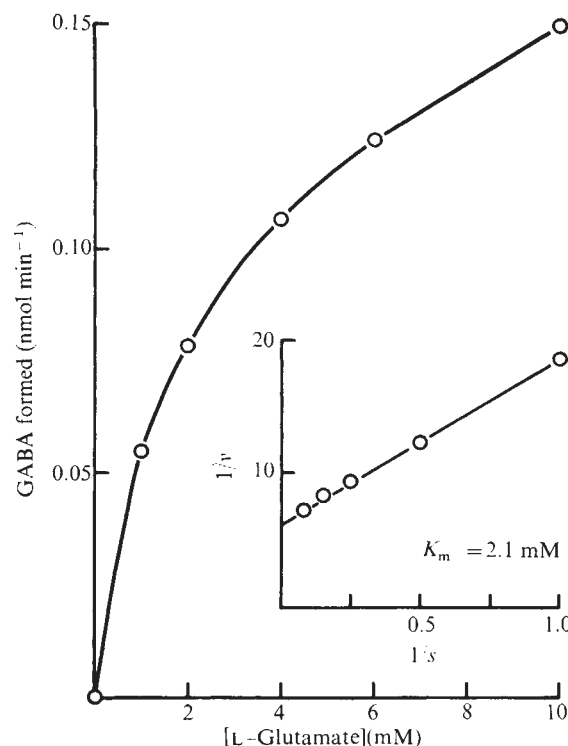


Fig. 2 GABA biosynthesis in pike olfactory nerve as a function of glutamate concentration. Assays were carried out as described in Table 4 using the specified concentration of glutamate.

The mammalian and lobster glutamate decarboxylase enzymes, which require pyridoxal phosphate as a cofactor, are inhibited by carbonyl-trapping reagents¹⁵. Table 3 shows that glutamate decarboxylase activity in the olfactory nerve extract of the pike is inhibited markedly by hydroxylamine. Semicarbazide, thiosemicarbazide and isoniazid are also inhibitory. Results were similar with the gar olfactory nerve (not shown). These studies support the hypothesis that the glutamate decarboxylase of the olfactory nerve requires a carbonyl-containing group for activity. After dialysis overnight against 2,000 volumes of buffer, pyridoxal phosphate stimulated GABA biosynthesis by 20%. As in the case of the mammalian and lobster enzymes¹⁵, the data suggest that the pyridoxal phosphate is rather tightly bound to the enzyme.

Mouse brain glutamate decarboxylase is inhibited by chloride¹⁶, while the mouse kidney enzyme⁹ (GAD II) and the lobster inhibitory nerve enzyme are not¹⁷. The data from Fig. 1

Table 2 Levels of GABA and its transaminase in tissues of gar and pike

Tissue	GABA (nmol g ⁻¹)		GABA transaminase (nmol min ⁻¹ g ⁻¹)	
	Gar	Pike	Gar	Pike
Olfactory nerve	0.55	0.49	46.3	24.1
Brain	0.74	0.75	87.9	50.3
Gill	< 0.02	< 0.02	34.4	7.6
Visceral fat	< 0.02	0.03	11.0	9.8
Gut	< 0.02	0.26	25.1	11.6
Skeletal muscle	NS	NS	22.1	NS

To assay GABA transaminase, tissues were homogenised in buffer *A*. To remove low molecular weight endogenous substrates, the extracts were dialysed against 100 volumes of buffer *A* for 1 h, against buffer *A* containing 100 mM KCl for 4 h and 12 h and against buffer *A* again for 1 h (all at 4° C) before assay. The GABA dependent conversion of labelled α -oxoglutarate into glutamate was measured¹⁴ and the labelled glutamate was resolved from precursor by paper electrophoresis.

NS, not studied.

Table 3 Inhibition of pike olfactory nerve glutamate decarboxylase by carbonyl-trapping reagents

Addition	Activity (pmol ¹⁴ C-GABA formed)
Control	35.9
Hydroxylamine, 0.2 mM	0.2
Isoniazid, 50 mM	4.8
Thiosemicarbazide, 12.5 mM	10.8
Semicarbazide, 50 mM	1.5

The assays were carried out as described in Table 1 using 10 µl extract (11 mg protein ml⁻¹) for a 15 min incubation at 37° C in the presence of the specified reagent.

show that sodium and potassium chloride inhibit the decarboxylase activity of the olfactory nerve. The KCl consistently inhibited activity more than NaCl, and parallel results were obtained with the gar olfactory nerve homogenate (not shown).

The olfactory nerve decarboxylase activity exhibits saturation kinetics (Fig. 2). The apparent K_m for the pike decarboxylase is 2.1 mM (Fig. 2) and that for the gar, 2.6 mM. Because crude dialysed homogenates were used, these values are not significantly different. The pH optimum for both the pike and gar enzymes, using 50 mM potassium phosphate buffer, is between 7.0 and 7.4.

In vertebrate neurones, the activity of the enzymes which catalyse neurotransmitter biosynthesis, and the neurotransmitter content is greatest in the synaptic region. Subcellular fractionation has shown that the biosynthetic enzyme activity and the content of acetylcholine¹⁸⁻²⁰, GABA²¹ and noradrenaline^{22,23} is greatest in the pinched off nerve ending, or synaptosome fraction. GABA and glutamate decarboxylase were measured in segments of the gar and pike olfactory nerves, cut progressively from the rostral to caudal ends. The enzyme activity, and the GABA content, show a rostral-to-caudal gradient with the greatest quantity nearest the synaptic region. To avoid adventitious contamination by non-olfactory nerve neurones, the synaptic region itself was not included for assay in the most caudal segment.

In the olfactory nerve of the gar and pike, GABA has several possible, and not necessarily mutually exclusive, functions. First, it might be a substrate for oxidative metabolism¹⁵. For example, conversion to succinate semialdehyde followed by oxidation to succinate yields 3 ATP equivalents. This GABA-shunt pathway accounts for about 10% of O₂ consumption in rat brain¹⁵. Second, GABA and its biosynthetic enzyme might be associated with the non-neuronal Schwann cells of the olfactory nerve. GABA and glutamate decarboxylase are found in mammalian glial cells grown in tissue culture²⁴. Unlike the olfactory nerve decarboxylase, however, the glutamate decarboxylase found in gliomas (GAD II) is not inhibited by salt or by carbonyl trapping reagents⁸. Third, GABA might be taken up by the olfactory nerve from synapses in the olfactory bulb region as part of a GABA-inactivating mechanism such as for the Schwann cell and connective tissue in the lobster neuromuscular junction.

Table 4 Axonal-to-synaptic gradient of GABA and glutamate decarboxylase activity in olfactory nerve

	GABA (µmol g ⁻¹)		Glutamate decarboxylase (nmol min ⁻¹ g ⁻¹)	
	Gar	Pike	Gar	Pike
Rostral	0.37		43	
		0.14	67	29
	0.41 0.58		93	
Caudal		0.59		77
	0.82		111	

Assays were carried out in the rostral-to-caudal quarters of the gar and halves of the pike olfactory nerves as described in Tables 1 and 2.

Although very unlikely, based on current concepts of its function, GABA might be a sensory excitatory neurotransmitter. It has been implicated so far exclusively as an inhibitory transmitter agent. Ionophoresis of GABA on to cerebellar and cerebral neurones²⁶, neurones in the dorsal Dieter's nucleus¹⁷ and in the lobster neuromuscular junction²⁸, all produce inhibition. Furthermore, experiments with the olfactory bulb of the cat and rabbit are consistent with the notion that granular cell inhibition of the mitral cell is mediated by GABA^{29,30}. On the other hand, the olfactory nerve in these preparations excites the mitral cells.

The presence of GABA, and its biosynthetic enzyme, in an axonal-to-synaptic gradient in the olfactory nerve of two fishes (Table 4) makes it a neurotransmitter candidate at the synapse between the nerve and the post-junctional cell in these species. Further experiments showing that GABA is released during neuronal activity and that it has the same effect on the post-synaptic cells as the natural transmitter are required before this compound can be considered a neurotransmitter substance. Until such time as the function is established, GABA and glutamate decarboxylase activity cannot be used to map inhibitory pathways in the vertebrate central nervous system.

Since this report was submitted, Flock and Lam, using methodology similar to our own, reported GABA synthesis in the sensory hair cells of amphibian and fish inner ear sense organs and fish lateral line organs³¹. The proposed function of GABA in these sensory transducing hair cells parallels that considered here for the olfactory nerve. On the other hand, Margolis suggested that carnosine may be related to olfactory nerve transmission in mouse³².

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Regulation of striatal acetylcholine concentration by dopamine receptors

IMPAIRMENT of the coordinated activities of cholinergic and dopaminergic neurones in the brain has been implicated in the pathophysiology of Huntington's chorea, drug-induced dyskinesias, Parkinsonism, schizophrenia and manic-depressive psychosis¹⁻⁶. Physostigmine, a drug which increases brain acetylcholine by inhibiting acetylcholinesterase, has been reported to improve the neurological status of patients with Huntington's chorea, tardive dyskinesias and L-dopa-induced dyskinesia⁷⁻⁹. The hyperkinetic involuntary movements in these disorders are thought to be related to a denervation hypersensitivity of dopamine receptors in the striatum¹⁰. On the other hand, in diseases with decreased dopaminergic activity, such as idiopathic and phenothiazine-induced Parkinsonism, intravenous injection of small doses of physostigmine exacerbated rigidity and tremors^{11,12}. A similar aggravation of Parkinsonian-like symptoms by physostigmine can be observed in rats pretreated with phenothiazines or reserpine and in dogs pretreated with intracisternal 6-hydroxydopamine^{13,14}. L-Dopa blocks the adverse neurological effects of physostigmine in Parkinsonian patients and in animals pretreated with reserpine or 6-hydroxydopamine¹¹⁻¹⁴. These observations suggest that the response to central cholinergic stimulation depends on the functional activity of inhibitory dopamine receptors in the striatum. The diminished striatal dopamine receptor stimulation in Parkinsonism¹⁵ may secondarily produce cholinergic hyperactivity in the striatum; the converse may exist in dyskinetic and choreiform disorders. Striatal dopamine receptors could regulate cholinergic activity by modulating intrastriatal acetylcholine synthesis and release by cholinergic neurones. We have now tested this hypothesis by investigating the effect of dopamine receptor agonists and antagonists on striatal acetylcholine concentration.

Male Sprague Dawley rats (180–200 g) were kept under constant diurnal lighting and temperature conditions for at least one week before use. Animals were killed at approximately the same time (0900–1200h) of day. All drugs were administered intraperitoneally. Control rats received an equal volume of

vehicle. Rats were decapitated at various times (Table 1) after the injection of apomorphine (10 mg kg⁻¹), trivastal (10 mg kg⁻¹), *d*-amphetamine (10 mg kg⁻¹), chlorpromazine (15 mg kg⁻¹), haloperidol (10 mg kg⁻¹) and pimozide (10 mg kg⁻¹). L-Dopa (100, 300, 600 mg kg⁻¹) was administered 1 h after MK 486 (α -methyl dopa hydrazide, 25 mg kg⁻¹) and the rats were killed 1 h after the second injection.

The brains were removed from the skull and placed immediately in cold pentane (–5° C). The striatum was dissected by the method of Glowinski and Iverson¹⁶. Bilateral striata from two rats were homogenised together in 0.4 N perchloric acid (4° C) containing propionylcholine iodide as an internal standard. Acetylcholine was extracted and assayed by gas-chromatography^{17,18}, modified as described previously¹⁹.

The concentration of acetylcholine in the striatum had increased significantly 0.25, 0.5 and 1 h after the administration of apomorphine, trivastal and *d*-amphetamine (all $P < 0.001$, Table 1). The maximum increase with apomorphine and trivastal was 49% and 83% respectively after 0.5 h and for *d*-amphetamine 33.6% after 1 h. Chlorpromazine, haloperidol and pimozide significantly (all $P < 0.001$) decreased striatal acetylcholine concentration 0.25, 0.5 and 1 h after injection. The maximum reduction with chlorpromazine and pimozide was 43% and 51.2%, respectively, after 0.25 h and with haloperidol 57.9% after 30 min. A significant reduction in acetylcholine concentration persisted for 4 h after injection of pimozide and for 16 h after injection of haloperidol (Table 1). L-Dopa (100, 300 and 600 mg kg⁻¹) significantly ($P < 0.001$) increased acetylcholine concentration in the striatum of rats pretreated with the peripheral dopa decarboxylase inhibitor MK 486 (Fig. 1). MK 486 or L-dopa, when administered alone had no significant effect on striatal acetylcholine concentration.

Dopamine, released from nigro-striatal nerve endings, reduced striatal neuronal discharge frequency^{20,21}. Since the striatum contains the highest concentration of acetylcholine²² in the brain, it is possible that intrastriatal cholinergic neurones which contain dopamine receptor sites are inhibited by dopamine. Our results support this hypothesis. Dopamine receptor agonists like apomorphine, trivastal, *d*-amphetamine and MK 486+L-dopa (which increases brain dopamine) may inhibit striatal cholinergic activity, which would reduce the release of acetylcholine from cholinergic neurones and produce the observed increase in striatal acetylcholine concentration. In contrast, dopamine receptor antagonists such as chlorpromazine, haloperidol and pimozide would increase discharge frequency of striatal cholinergic neurones, increase the release of striatal acetylcholine and produce the observed decrease in striatal acetylcholine concentration. The decrease in striatal acetylcholine concentration is not likely to be due to a central anticholinergic action of these neuroleptic drugs since chlorpromazine actually enhances the tremorigenic effect of physostigmine both in man and animals^{12,13}. Anticholinergic drugs which decrease brain acetylcholine also reduce the tremorigenic action of physostigmine²³. Furthermore, we have observed that pretreatment with L-dopa (300 mg kg⁻¹) significantly blocked the chlorpromazine-induced

Table 1 Effects of drugs on concentration of acetylcholine in the striatum

Drug	Dose (mg kg ⁻¹)	Time after injection						
		0 h	0.25 h	0.5 h	1 h	4 h	16 h	24 h
Apomorphine	10	29.6 ± 1.0	41.2 ± 1.1	44.1 ± 2.3	38.2 ± 2.8	25.0 ± 1.3	—	—
Trivastal	10	31.1 ± 1.6	52.2 ± 0.8	57.0 ± 4.3	48.3 ± 5.2	29.4 ± 2.2	—	—
<i>d</i> -Amphetamine	10	30.9 ± 0.4	34.6 ± 1.3	41.3 ± 1.8	42.9 ± 1.2	29.6 ± 0.7	—	—
Chlorpromazine	15	30.8 ± 1.1	17.6 ± 2.6	20.0 ± 1.3	23.6 ± 1.8	28.6 ± 1.8	—	—
Haloperidol	10	30.9 ± 0.6	17.4 ± 1.5	13.0 ± 1.4	16.7 ± 0.7	18.0 ± 0.8	24.3 ± 1.0	25.8 ± 1.2
Pimozide	10	29.3 ± 1.1	14.3 ± 1.4	14.5 ± 1.8	17.5 ± 1.3	18.5 ± 1.0	30.6 ± 0.8	—

Figures are nmol g⁻¹.

Acetylcholine concentration in the rat striatum after the intraperitoneal administration of either dopamine receptor agonists or antagonists. Each value is the mean ± s.e. of 4–15 observations.

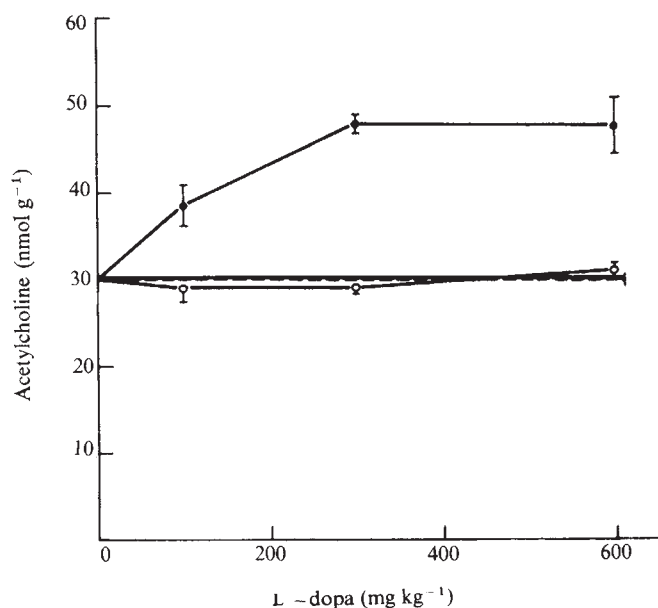


Fig. 1 Effect of various doses of L-dopa on striatal acetylcholine concentration in control and MK 486-treated rats. Each point is the mean $\pm$ s.e. of four to twelve determinations. — — —, Control; — — —, MK 486; ○, L-dopa; ●, MK 486 + L-dopa.

decrease in striatal acetylcholine concentration, indicating that the changes in acetylcholine concentration are mediated by functional changes in the dopamine receptors.

Our results are consistent with the demonstration by Stadler *et al.*²⁴ that chlorpromazine increased acetylcholine release from the caudate nucleus of the cat, and that this release was blocked by apomorphine. However, Jones *et al.*²⁵ found no alterations in acetylcholine release during superfusions of the ventricular surface of the caudate nucleus in cats treated with either L-dopa or amphetamine. Reserpine, a drug which decreases brain dopamine, also lowers striatal acetylcholine and this effect is reversed by pretreatment with L-dopa²⁶.

In idiopathic and phenothiazine-induced Parkinsonism there is a decrease in striatal dopaminergic activity and an apparent increase in cholinergic activity. Further enhancement of cholinergic activity by the injection of acetylcholine into the basal ganglia of Parkinsonian patients was found to increase tremor on the contralateral side, while anticholinergic drugs reduced the tremor²⁷. Excessive discharge frequency of striatal cholinergic neurones associated with increased release of acetylcholine and decreased acetylcholine concentration may be causally related to the Parkinsonian side effects of the neuroleptic drugs. This mechanism would account for the therapeutic effectiveness of anticholinergic drugs in drug-induced Parkinsonism.

A stimulatory effect of neuroleptic drugs on cholinergic neurones could also be relevant to their therapeutic effectiveness in schizophrenia. Several clinical studies have demonstrated that drugs which stimulate central cholinergic neurones improve schizophrenic symptomatology. Cholinomimetic compounds like arecoline and physostigmine have been observed to produce symptomatic improvement in schizophrenic patients^{28,29}. On the other hand, anticholinergic drugs, L-dopa and methylphenidate aggravate the symptoms of schizophrenia³⁰⁻³². Physostigmine reversed the intensification of the schizophrenic psychosis produced by methylphenidate³³. The anti-schizophrenic action of phenothiazine drugs, haloperidol and pimozide could be related to their ability to block an excessive dopaminergic inhibition of cholinergic neurones in the brain.

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Quantitation of adrenaline in rat brain nuclei and areas by mass fragmentography

THE existence of adrenaline (A) in the mammalian central nervous system (CNS) was first demonstrated by von Euler¹ and Holtz². Subsequently, many reports have documented the presence of whole brain A in mammals and amphibia. In amphibia adrenaline is the major CNS catecholamine³, being about four times greater than noradrenaline⁴, while in mammals, adrenaline is only a small fraction (4-22%) of the total noradrenaline content^{5,6}. These data in mammals

could indicate either adrenaline uptake from the periphery or a very discrete adrenaline pathway possibly with a high turnover rate. We have quantitatively measured adrenaline in some specific nuclei of the rat brain, using the highly sensitive and specific technique of gas chromatography-mass spectrometry (GC-MS)⁷.

In our preliminary experiments, 15 brain nuclei were dissected and assayed for their catecholamine content. Male rats (125–175 g) were killed by cervical fracture and the brain rapidly removed and frozen in powdered dry ice. The frozen brains were serially sectioned (400 μ m, knife angle 30° to the brain axis) in a microtome cryostat (−5° C) and the nuclei located and punched out with a hollow steel tube (0.5–1.2 mm internal diameter), using the atlases of König and Klippel⁸, Zeman *et al.*⁹, and Wunscher *et al.*¹⁰ as a guide. The catecholamines were extracted into 100 μ l 0.1 M formic acid 50 mM ascorbic acid; 60 μ l was taken to dryness in the presence of internal standards and the dry residue reacted with pentafluoropropionic anhydride⁷. After 30 min reaction at 60° C, the excess acylating reagent was removed, and the acylated catecholamines redissolved in 10 μ l ethylacetate and 2 μ l of this solution was analysed by GC-MS (LKB 9000) using a column packed with 3% SE 54 on Chromasorb G. By using the combined instrumentation of GC-MS it is possible to quantitatively measure the catecholamines without having any interference from other compounds. The catecholamines are initially resolved on the GC, for example, the retention times for noradrenaline, adrenaline, dopamine and epinephrine are 370, 425, 510 and 600 s respectively. At the retention time for adrenaline (425 s) the MS is focused at *m/e* 190 which is the base peak (100%) for adrenaline. Since the fragment *m/e* 190 is approximately 60% of the total ions produced, the sensitivity of the detection is extremely high; less than 10 fmol (10×10^{-15}) adrenaline can be measured.

Fifteen different rat brain nuclei were initially (triplicate determinations) analysed individually for their catecholamine content. Of these regions all contained noradrenaline while only five contained adrenaline. The five regions which contained adrenaline were: the ventral lateral N. reticularis (A₁); the cerebellum at the level of the genu N. facialis, including the region from the lateral edges of the rv ventricle, juxtaposed to and possibly including the N. superior and N. principalis vestibularis and fibrae vestibulo cerebellares; the N. locus coeruleus; the habenula region and the periventricular area of the anterior hypothalamus including the nuclei and regions listed in Table 1. In these five regions (Table 2) the percentage adrenaline ranged from 1.0 to 6.5 of the noradrenaline. The highest concentration of adrenaline, 5.7 pmol mg⁻¹ protein was found in the periventricular area of the hypothalamus, while the highest adrenaline concentration (as % noradrenaline) was found in the cerebellum.

The periventricular areas of the hypothalamus were further dissected into four subregions and assayed for adrenaline. Adrenaline was found in all four regions with the highest concentrations being found in the N. periventricularis hypothalami, and the area infundibulum and nucleus arcuatus (Table 1).

Table 2 Adrenaline concentrations of five rat brain nuclei and areas*

Area or nuclei	Adrenaline (pmol mg ⁻¹ protein)	Noradrenaline (pmol mg ⁻¹ protein)	Adrenaline Noradrenaline $\times 100$
N. Reticularis lat (A ₁)	1.3 $\pm$ 0.33	75 $\pm$ 15	1.7
Cerebellum	1.1 $\pm$ 0.03	17 $\pm$ 4.4	6.5
N. Locus coeruleus	1.4 $\pm$ 0.16	140 $\pm$ 20	1.0
Habenula	1.2 $\pm$ 0.16	57 $\pm$ 3.1	2.1
Periventricular area of the hypothalamus	5.7 $\pm$ 1.1	160 $\pm$ 23	3.6

*Mean $\pm$ s.e. of four determinations.

These preliminary data support the proposal of Hokfelt *et al.*¹¹ that a neuronal network containing adrenaline is present in rat brain. They base this conclusion on their immunocytochemical localisation of phenylethanolamine-N-methyltransferase (PNMT) in neurones. They reported a total of 17 areas or nuclei in rat brain which gave a positive PNMT reaction. We have not examined all of these areas as yet but some of our preliminary findings reported here agree with the results of the PNMT reaction; N. reticularis lat (A₁) contains cell bodies reacting to PNMT and the locus coeruleus and periventricular areas of the hypothalamus contained PNMT positive terminals. Saavedra *et al.*¹² have recently reported on the biochemical measurement of PNMT activity in rat brain nuclei. The PNMT activity was unevenly distributed and the biochemical localisation of the enzyme agreed well with the histological data of Hokfelt *et al.* There does not, however, seem to be good agreement between the PNMT activity and adrenaline concentrations, that is A₁ region 41.5, and N. paraventricularis 15.0, PNMT activity against adrenaline concentration of 1.3 and 4.0 pmol mg⁻¹ protein, respectively.

In these preliminary experiments adrenaline has been measured in a few discrete areas of the rat brain, and found to be present in only certain regions. At this point we cannot postulate that the adrenaline is confined to the PNMT neuronal network, additional studies are presently being done to test this hypothesis. It is likely, however, that adrenaline is contained in neurons which presumably function independently of the noradrenaline and dopamine neuronal systems.

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Table 1 Adrenaline concentrations in the periventricular areas of the hypothalamus

	Adrenaline (pmol mg ⁻¹ protein)
N. Paraventricularis (filiformis) pars magnocellular	2.0 $\pm$ 0.24
N. Paraventricularis (filiformis) pars parvocellularis	1.6 $\pm$ 0.18
N. Periventricularis (hypothalami)	4.0 $\pm$ 0.40
Area Infundibulum + N. Arcuatus	3.4 $\pm$ 0.95

*Mean $\pm$ s.e. of three determinations.

Effect of reserpine on cationic contents of rat brain

PREPARATIONS of the plant *Rauwolfia serpentina* have been used in India for centuries as a calming drug. Reserpine is the principal alkaloid of this plant and its effect on the central nervous system (CNS) has been studied extensively. The most important biochemical effect of reserpine on the CNS found *in vivo* is the release of noradrenaline, 5-hydroxytryptamine and dopamine from the amine storage sites in brain so that they are more susceptible to degradation by monoamine oxidase¹. In synaptosomal preparations, reserpine has been shown to inhibit the uptake of noradrenaline and 5-hydroxytryptamine². Prolonged reserpine treatment depletes these amines from the brain¹. Amine depletion is thought to be due to changes in the permeability of neuronal membranes³.

Tetrodotoxin and local anaesthetics increase the glycolysis of cerebral cortex slices⁴ and this prompted us to investigate a number of neurotropic agents including reserpine. Studies with reserpine revealed that this drug can cause an increase in the potassium content of cerebral tissue both *in vitro* and *in vivo*. In view of the important role played by cations in the regulation of brain metabolism⁵, the effect of reserpine on the potassium contents is of great interest and we are surprised that such measurements have not been made previously.

Rat cerebral cortex slices were prepared and incubated as described earlier^{4,5} and anaerobic glycolysis, cationic contents, ²²Na transport and respiration were measured. For the *in vivo* experiments rats were given reserpine (2 mg kg⁻¹, Ciba) or saline intraperitoneally for 5 d after which they were killed, the brains quickly removed, weighed and protein-free extracts prepared for cation analysis by a Coleman flame photometer.

The *in vitro* experiments, details of which will be published elsewhere, showed that during anaerobic conditions, 12–120 µM reserpine greatly enhances the rate of glycolysis by adult rat and guinea pig cerebral cortex slices. The maximum effects observed were up to 3–4 times the normal rate. It was thought possible that reserpine may exert this effect indirectly by amine release. Experiments carried out with biogenic amines showed that unlike reserpine, these amines have no significant effect on anaerobic glycolysis. It was therefore concluded that reserpine may also exert its effect on the anaerobic glycolysis by increasing K⁺/Na⁺ ratio, as is the case with tetrodotoxin and local anaesthetics. This was found to be true and reserpine had a significant effect in increasing the K⁺ contents of the cerebral cortex slices incubated under anoxic conditions, the increase being up to 34 µeq per g initial wet weight of the tissue as compared to 17 µeq per g initial wet weight for the controls.

Other experiments further demonstrated that 12 µM reserpine may affect the cationic movements. It was found that the respiration of rat cerebral cortex slices, stimulated by 10 µM protoveratrine, was suppressed by 12 µM reserpine. These effects were much more evident in a Ca²⁺-free Krebs–Ringer phosphate medium. ²²Na movement was also suppressed by reserpine in these conditions.

A most interesting effect of reserpine was observed *in vivo* (Table 1). It was found that chronic reserpine administrations results in an increase in the K⁺ contents of brain of more than

25% while the Na⁺ contents are not affected. The cationic contents described here were determined in the whole brain. It is possible that most of the K⁺ increase occurs intracellularly (and primarily in the neurones as compared to the glia), since very little extracellular space is present in brain.

The effect of reserpine on the amine uptake presumably results from changes in the permeability of neuronal membrane with a concomitant increase in the steady state level of K⁺. Our results have wide implications. Sedation induced by reserpine is thought to result from a decrease in the quantity of monoamines¹. High cerebral potassium in reserpinised animals may cause neuronal hyperpolarisation resulting in the potentiation of sedative effects. It has been argued that barbiturates and other sedatives may primarily act by inhibiting impulse conduction rather than amine metabolism⁷.

Our observations also raise the question whether an increase in the level of cerebral K⁺ is the primary effect of reserpine or is caused indirectly due to other central effects. The former possibility seems to be true. The uptake of amines by nerve endings is dependent on the presence of Na⁺ (ref. 8), and the mechanism is activated by excitation and thus depolarisation¹⁰. Membrane affinity for neurotransmitters is affected by nervous stimulation⁹. Moreover, K⁺ has been shown to compete with Na⁺ for the Na⁺-dependent uptake of monoamines^{10,11}. Almost 80% of noradrenaline released at the synapse is inactivated by the uptake mechanism⁹. It seems that the increase in neuronal K⁺ affects the uptake of amine by storage granules, presumably by competing with Na⁺ (refs 10 and 11). Modulation of membrane affinity to neurotransmitters, therefore, seems to be involved in the action of reserpine. Further investigations are in progress to examine these possibilities.

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Table 1 Effect of chronic reserpine administration on the Na⁺ and K⁺ contents of rat brain

Treatment	K ⁺ (µeq per g fresh weight)	Na ⁺ (µeq per g fresh weight)
Control (12)	122.7 ± 5.7	48.5 ± 3.0
Experimental (12) (Reserpine, 2 mg kg ⁻¹ d ⁻¹ for 5 d)	157* ± 4.7	49.3† ± 2.5

K⁺ and Na⁺ contents were determined as given in the text. Values obtained, and their standard deviations are given. Number of observations are shown in parentheses.

*P < 0.001, highly significant.

†P > 0.4, not significant.

S-100 protein in synapses of the central nervous system

THE brain-specific S-100 protein¹ is predominantly localised in glial cells in the central nervous system and is synthesised by glial cells in tissue culture^{2–3}. S-100 is, however, also present in neurones^{6–9}, located in both the neuronal perikaryon and the nucleus^{8,9–11}. It has been proposed that the protein is transported along the axon¹⁰ although the presence of S-100 in the Schwann cell^{12,13} calls for confirmation of an intra-axonal localisation of the protein. Approxi-

mately 90% of the S-100 in the brain is easily soluble and extracted by water¹⁴⁻¹⁵, but an additional small amount can be extracted from the particulate matter with *n*-pentanol. Rusca *et al.*¹⁴ showed a further release 5-7% of the S-100 from whole beef brain, and Haglid *et al.*¹³ extracted 7% from human grey matter and 3% from white matter with *n*-pentanol. The percentage of total S-100 which remained bound to the particulate matter after water extraction was considerably higher in neuronal perikarya than in glial cells (manuscript in preparation). We have investigated the ultrastructural location of the relatively large amounts of membrane-bound neuronal S-100.

White albino rabbits, weighing 1.5-2 kg, were killed after mebumal anaesthesia and decapitated. Parts of the CNS were dissected fresh, immersed in ice-cold 0.1 M cacodylate buffer, pH 7.4, and cut to 10-20 μ m thick sections on a Vibratom (Oxford Instrument). In other experiments, the brains were fixed by intra-aortal perfusion with 2% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, and rinsed in the same buffer after a fixation time of 5 min. All solutions in contact with the tissue slices contained 2.5 mM EDTA or EGTA. The fresh and prefixed sections were incubated for 30 min in 0.1 M cacodylate buffer, containing antiserum against S-100 coupled with peroxidase. They were then extensively rinsed, and thereafter fixed in 2.5% glutaraldehyde and 2% formaldehyde in cacodylate buffer.

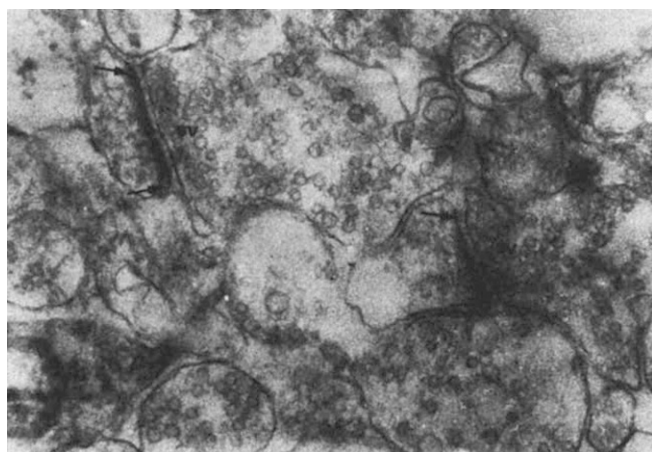


Fig. 1 Electron micrograph of synapses in the frontal cortex of an adult albino rabbit. The tissue piece was treated with rabbit antiserum to beef S-100 protein coupled to peroxidase as described in the text. The postsynaptic membranes (arrow) show a strong peroxidase activity. No activity is seen in the synaptic cleft or in the synaptic vesicles. The activity of the presynaptic membrane is about the same as in other neuronal membranes. $\times 42,750$.

Cerebellar glomerular complexes were isolated by Ficoll gradient centrifugation according to A.H., H.-A.H., and Å. Sellström (in preparation). The glomerular suspension was incubated at room temperature for 10 or 30 min with the peroxidase-coupled antiserum against S-100, sedimented and treated like the tissue sections. All material was then thoroughly rinsed and incubated in a cacodylate buffered solution of 3,3-diaminobenzidine and hydrogen peroxide¹⁶. The samples were rinsed, postfixed in osmium tetroxide, dehydrated and embedded in Epon. Thick sections were cut on an ultramicrotome (LKB Ultratome III) and examined in a phase contrast microscope. Selected areas were sectioned for examination in an electron microscope (Siemens 1 A). They were either unstained or double-stained with uranyl acetate and lead citrate. The purification of beef S-100 and the production of anti-beef S-100 rabbit antiserum, as well as the monospecificity of the antibodies in the serum were tested by microcomplement fixation, immunoelectrophoresis and other electrophoretic methods^{13,15}. The peroxidase (Sigma Type VI) was coupled to the anti-

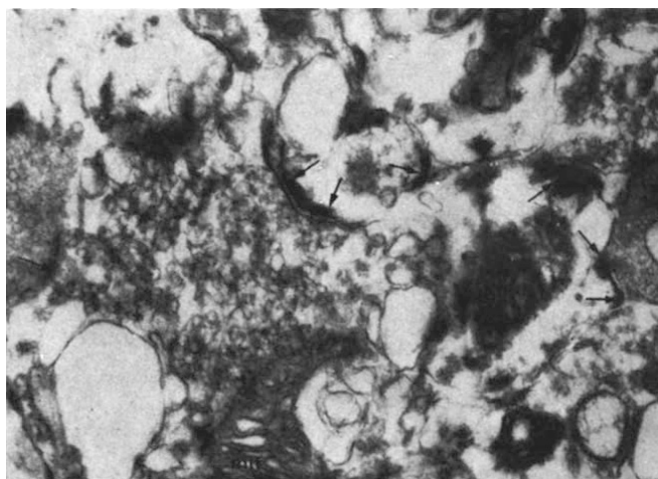


Fig. 2 Electron micrograph of an isolated cerebellar glomerulus after treatment with rabbit antiserum to beef S-100 protein coupled with peroxidase. Note the dark reaction products indicating peroxidase activity, that is, localisation of S-100 protein at the postsynaptic membrane (arrow). The other neuronal membranes have a lower level of activity than the postsynaptic membrane. The mitochondria (mit) may show nonspecific activity. $\times 31,950$.

serum according to Avrameas¹⁷. The peroxidase-coupled anti-beef S-100 rabbit serum was separated from free peroxidase by G-75 Sephadex column chromatography. The specificity of the peroxidase-coupled anti-beef S-100 rabbit serum reactions was checked by the use of a control antiserum which had been repeatedly (12 times) absorbed with purified beef S-100 protein. Test and control antisera were diluted and treated in an identical manner. The completeness of the absorption was tested by microcomplement fixation against the original antigen. The final dilution of antiserum was titrated by chessboard until maximal test-control difference in peroxidase activity was observed.

Specific peroxidase activity was seen in most synaptic regions of the cerebral and cerebellar cortex. Heavy precipitates were localised exclusively on, and immediately interior to, the postsynaptic membrane (Fig. 1). No activity was seen in the synaptic cleft. All other parts of the neuronal membrane—including the presynaptic membrane—had a low or moderate but specific peroxidase activity. The synaptic vesicles, endoplasmic reticulum, filaments, microtubules and nuclei did not show any significant peroxidase activity. Some mitochondria showed peroxidase activity also after treatment with antisera absorbed repeatedly with the antigen protein S-100. Such nonspecific peroxidase activity has previously been described¹⁷. Neither peroxidase-coupled rabbit antisera directed against other soluble or particulate brain proteins, nor free peroxidase showed any localisation to the postsynaptic membrane.

In order to investigate whether the distribution of peroxidase activity was affected by penetration barriers, we examined suspensions of isolated cerebellar glomeruli, which consist of clusters of synaptic contacts. Again, intense peroxidase activity was observed at the postsynaptic membrane (Fig. 2). A different pattern was observed in the synaptic terminals of the rods and some bipolar nerve cells in the rabbit retina (Fig. 3). An intense reaction was seen in the synaptic ribbon and in both the pre- and postsynaptic membranes in these composite synaptic terminals. In retinal synapses of more conventional type the peroxidase reaction was confined to the postsynaptic region, as in the cerebral and cerebellar synapses.

The S-100 protein visualised by peroxidase-coupled anti-beef S-100 rabbit serum is presumably particulate bound, as fixed and fresh slices gave a similar picture—the fresh slices were subjected to buffer solution in multifold excess which makes the soluble S-100 protein easily diffusable. The

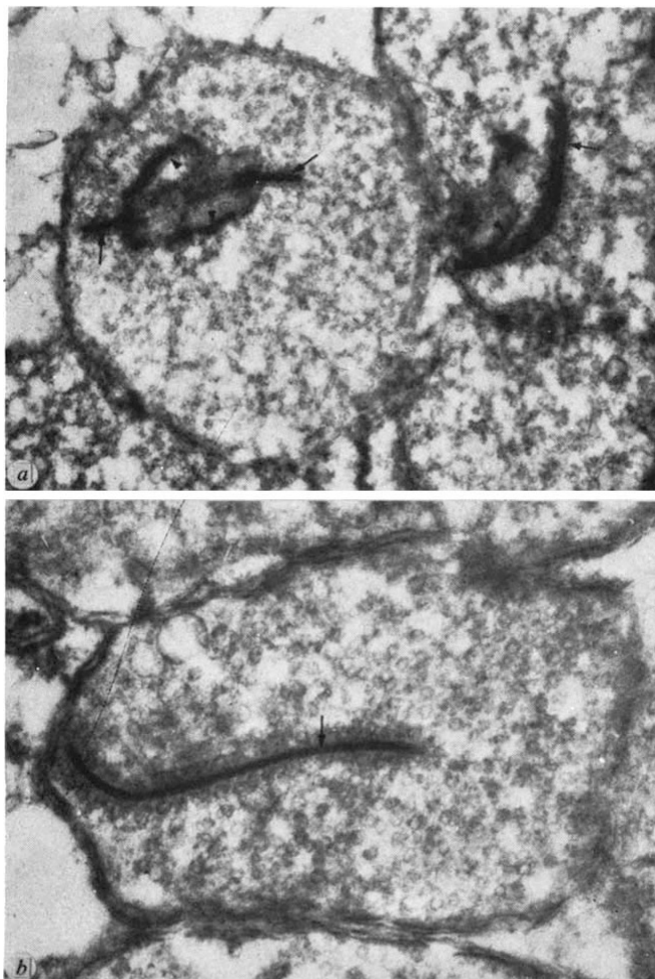


Fig. 3 Electron micrograph of synaptic terminals in the outer plexiform layer of the rabbit retina treated as in Fig. 1. Dark deposits, indicating peroxidase activity, are seen in the synaptic ribbon (arrow) and at the synaptic contacts with bipolar nerve cells (arrowhead) and also, although at a lower level, in the neuronal membrane. The synaptic vesicles show mainly background activity. *a*, $\times 21,300$; *b*, $\times 22,720$.

soluble form of S-100 does not bind the subcellular particulate material in the presence of EDTA (B. W. Moore, communicated at NATO Advanced Study Institute meeting in Cortona, Italy, 1971).

The functional role of the S-100 protein is so far unknown. Its interaction with Ca^{2+} ions¹⁸ and with the passage of monovalent and divalent cations across artificial lipid membranes¹⁹ may lead to speculations about its function. We here present data which indicate a specialised synaptic localisation of the S-100 protein. This might imply a functional interrelation between a glial brain-specific protein and synaptic function, since so far there is no evidence that this protein is synthesised in neurones.

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Dihydrostreptomycin accumulation in *E. coli*

DIHYDROSTREPTOMYCIN is an antibiotic composed of streptidine, dihydrostreptose and N-methyl-L-glucosamine joined by two glycosidic bonds. It differs from streptomycin only in that the aldehyde group on the middle residue (streptose) is reduced. Both compounds have similar biological actions^{1,2} but their accumulation by bacterial cells has been little studied. Passive diffusion is often assumed to be responsible, although Mitchell thought it improbable that a molecule such as streptomycin passes through a lipid layer³. Anand *et al.* proposed a mechanism in two phases⁴. First, a small amount of antibiotic would enter by passive diffusion regardless of an intact lipid layer and affect protein synthesis so as to cause deterioration of membrane integrity. Then more rapid accumulation would follow by passive diffusion through the disrupted lipid barrier. Alternatively, Hurwitz *et al.* showed streptomycin accumulation to be sensitive to chloramphenicol during initial exposure and insensitive after several minutes of exposure. They suggested facilitated diffusion, with entry of the antibiotic depending on a streptomycin-induced protein⁵.

Carlson and Bockrath found dihydrostreptomycin accumulation to vary depending on whether bacterial cells grew in a glucose-defined medium or in nutrient broth⁶. Accumulation was roughly fifty times more efficient in nutrient broth cultures or with cells recently transferred from nutrient broth in the defined medium. After an apparent lag of a few minutes, the kinetics of accumulation were generally linear with time for at least 30 min. Anand *et al.* used cells growing in defined medium and Hurwitz *et al.* used cells growing in nutrient broth. We have repeated the observation of a chloramphenicol-insensitive phase of accumulation and have found that cells growing in nutrient broth accumulate antibiotic during this phase by a transport mechanism involving a positive contribution from cellular metabolism.

A culture of *Escherichia coli* B growing exponentially

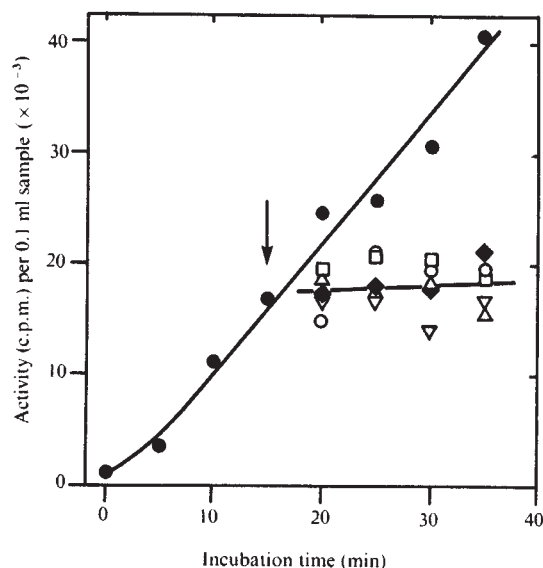


Fig. 1 Inhibition of ^3H -dihydrostreptomycin accumulation. ^3H -dihydrostreptomycin sesquisulphate ($2 \mu\text{g ml}^{-1}$, Amersham/Searle, specific activity 2.1 mCi mg^{-1}) was added at zero time to an exponentially growing culture of *E. coli* B in Difco nutrient broth (4.8×10^7 bacteria ml^{-1}). At 15 min (arrow) aliquots were removed to separate cultures for specific perturbations during continued incubation: ∇ , transferred to ice bath; $\blacklozenge$, potassium arsenate (10 mM); Δ , mercuric chloride (0.2 mM); $\square$, N-ethyl maleimide (1 mM); and $\circ$, iodoacetate (2 mM). All incubations were at 37°C (except ∇) with aeration. Accumulated radiolabelled antibiotic was determined during incubation time: 0.1 ml samples were diluted $\times 100$ into buffer⁶ at room temperature, filtered on to glass fibre filters, rinsed twice with 10 ml of buffer, dried and quantitated by liquid scintillation counting.

in Difco nutrient broth was challenged at zero time with ^3H -dihydrostreptomycin (^3H -dhSm, $2 \mu\text{g ml}^{-1}$). Chloramphenicol (20 or $200 \mu\text{g ml}^{-1}$) inhibited ^3H -dhSm accumulation when added at zero time but had little effect if added 10 min later. The rate of accumulation when chloramphenicol was added at zero time (just before the addition of ^3H -dhSm) was very small and showed no tendency to increase. Repeated experiments found chloramphenicol to be a good inhibitor if added at zero time, a moderate to good inhibitor of accumulation if added 5 min after the initial exposure to ^3H -dhSm, and moderate to poor if added after 10 min . Therefore, it seems that protein synthesis is of marginal importance to the accumulation of ^3H -dhSm by *E. coli* B once the cells have been exposed to dihydrostreptomycin for a period of 10 min .

Table 1 Perturbations of ^3H -dihydrostreptomycin accumulation

Challenge	Concentration (mM)	Accumulation (%)
Ice bath	—	0
Dinitrofluorobenzene	0.5	0
2,4-Dinitrophenol	2.0	0
Potassium cyanide	0.5	5
Mercuric chloride	0.2	8
Potassium arsenate	10.0	8
Iodoacetate	2.0	12
N-ethyl maleimide	1.0	21
Phenazine methyl sulphate	0.1	22
Potassium fluoride	5.0	47
Sodium fluoride	5.0	58
Amytal	0.1	58
Rotenone	0.1	79
Oxamic acid	0.5	114

Perturbations of ^3H -dihydrostreptomycin accumulation were determined as described in Fig. 1. In each case the indicated challenge was introduced after 10 or 15 min of accumulation. The effect is recorded as the slope of accumulation after the challenge relative to the slope of accumulation in the control.

Dramatic inhibition of accumulation may, however, be produced by cold and by several anti-metabolites added 10 or 15 min after the initial exposure to ^3H -dhSm. Figure 1 shows several examples of an abrupt cessation of ^3H -dhSm accumulation. The perturbations are not introduced until 15 min after initiation of exposure. The results of these experiments and several others are summarised in Table 1. Clearly they suggest a metabolism-dependent mechanism of ^3H -dhSm accumulation. Several inhibitors of metabolism can cause accumulation to stop and plateau regardless of a hypothetical deterioration of membrane integrity or a physiological development obviating the requirement for protein synthesis.

Anand *et al.*, who rinsed their samples with water, found toluene treatment of cells to cause an apparent influx of streptomycin (with an exogenous antibiotic concentration of $40 \mu\text{g ml}^{-1}$). In our experiments cells growing in nutrient broth accumulated large amounts of ^3H -dhSm (from an exogenous concentration of $2 \mu\text{g ml}^{-1}$); we rinsed individual samples with a buffer⁶ and found the addition of toluene then caused an apparent efflux of the antibiotic (Fig. 2). To ensure that the toluene treatment did not totally dissociate the cell structure or alter the filtration system to allow cells to pass through the filter, cells prelabelled with ^3H -uracil were also treated. In Fig. 2 the open symbols show this activity (labelled ribosomes and nucleic acid) to remain on the filter unless the cells were further treated with lysozyme.

An integral cellular membrane is therefore essential in our assay for accumulated ^3H -dhSm. Apparently antibiotic molecules which have already entered the cell do not

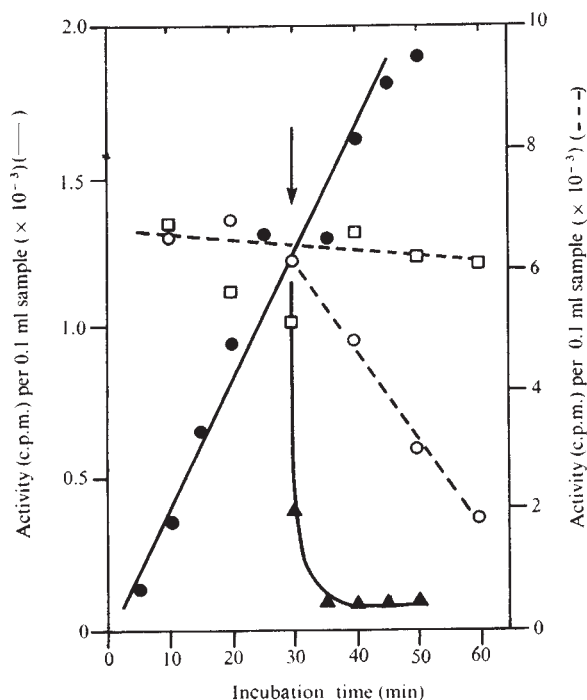


Fig. 2 Effects of toluene on the accumulation assay. ^3H -dihydrostreptomycin ($2 \mu\text{g ml}^{-1}$, Mallinckrodt, specific activity, 0.25 mCi mg^{-1}) was added at zero time to an exponentially growing culture of *E. coli* B in Difco nutrient broth (6×10^7 bacteria ml^{-1}). After 30 min 0.02 ml ml^{-1} toluene was added to one aliquot ($\blacktriangle$), with the remaining culture serving as a control ($\bullet$). A nutrient broth culture of *E. coli* B was also grown with ^3H -uracil ($1.5 \mu\text{Ci ml}^{-1}$) for several generations and divided into two aliquots at zero time. Dihydrostreptomycin (unlabelled), $2 \mu\text{g ml}^{-1}$, was added to both; one ($\square$) was treated with toluene, 0.02 ml ml^{-1} , at 30 min , and the other ($\circ$) was treated with toluene after 15 min and lysozyme, 0.4 mg ml^{-1} , after 30 min .

equilibrate rapidly with the rinse buffer unless the membrane is altered by an agent such as toluene. In consequence, it seems the normal membrane is not transparent to ^3H -dhSm and a brief period of exposure to ^3H -dhSm does not open the membrane.

Plotz *et al.* have shown that when normal cells are held in a water rinse apparent uptake is increased⁷. We found toluene-treated cells, similarly held in a water rinse containing a representative amount of ^3H -dhSm, to yield an even larger apparent uptake. We therefore doubt that toluene treatment of cells in culture necessarily allows ^3H -dhSm to rush in. More likely, the equilibrium of ^3H -dhSm between the medium and the cell volume is principally a function of the cation-exchange capacity of cell biomass⁸ and the intracellular concentration of competing cations. The apparent accumulation of antibiotic in toluene-treated cells would vary with the ionic composition of the rinse solution.

In summary, after a period of potentiation during which cellular uptake of ^3H -dhSm becomes refractory to inhibition by chloramphenicol, the accumulation of ^3H -dhSm is still sensitive to inhibitors of metabolism. Our assay for accumulation of antibiotic uses a buffer rinse which does not seem to remove accumulated ^3H -dhSm unless the integrity of the cell membrane is disrupted. These findings are consistent with an accumulation mechanism in *E. coli* for dihydrostreptomycin that involves either active transport or group translocation.

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nuclease to look directly at the inheritance of mtDNA nucleotide sequences. When any homogeneous DNA preparation is digested to completion by a site-specific restriction endonuclease, a characteristic set of fragments is produced. If the DNA is of low complexity, as in the case of simple viruses^{9,10} or mammalian mtDNA¹¹, separation of the restriction fragments on the basis of molecular weight by gel electrophoresis produces a characteristic 'fragment pattern' (Fig. 1). This pattern is characteristic of the original DNA molecule in much the same way that the tryptic fingerprint of a protein is characteristic of that particular protein.

We have studied the inheritance of mtDNA by isolating pure mtDNA from hybrids between two closely related mammalian species. The two parental mtDNAs are readily distinguishable by restriction enzyme cleavage analysis; analysis of the mtDNA from the hybrid animals yielded a fragment pattern characteristic of the maternal species. None of the DNA fragments specific for the paternal species were observed.

We obtained our material from common equine species: the horse (*Equus caballus*), the donkey (*E. asinus*), and the two reciprocal hybrids between them, the mule and the hinny. The two hybrids are defined as (1) ♀ horse × ♂ donkey → mule and (2) ♀ donkey × ♂ horse → hinny. Mules and hinnies may be of either sex but are generally sterile.

Mitochondria were partially purified from liver or heart tissue, and the DNA extracted from such preparations was centrifuged in CsCl-ethidium bromide gradients¹². Pure mtDNA was isolated from the supercoil band in such gradients. The purified mtDNAs were then digested with a restriction endonuclease isolated from *Haemophilus aegyptius*, endonuclease Z¹³ (endonuclease R-Hae III in the nomenclature of ref. 14), and the resulting digests were analysed by electrophoresis in 3.5% polyacrylamide gels. The DNA-containing bands were demonstrated by fluorescence after staining with ethidium bromide¹⁵.

The fragment patterns obtained from horse and donkey mtDNAs are clearly distinguishable (Fig. 1). We examined DNA from six horses, two full-sized (one of each sex) and four ponies. Their mtDNA cleavage patterns were almost identical. The two horse patterns were indistinguishable, and the pony patterns differed in the position of one or two of the lower molecular weight fragments. These minor intraspecies variations in the fragment pattern seem to be significant and will be discussed elsewhere (manuscript in preparation). mtDNA from two male donkeys yielded fragment patterns indistinguishable from each other, but clearly different from the characteristic horse pattern. But although the two species yield readily distinguishable fragment patterns, there appears to be considerable homology between the two patterns.

Cleavage analysis of mule mtDNA gives the maternal (horse) fragment pattern (Fig. 1). All three animals that we have examined (one male, two females) yielded the standard horse pattern. This could be explained in terms of a general phenomenon of maternal inheritance of the mtDNA. Alternatively, however, it could be a result of a species-specific loss of donkey mtDNA in horse-donkey hybrid cells. To distinguish between these possibilities we have analysed the mtDNA from a hinny. Again the maternal pattern was observed (a donkey pattern in this case, see Fig. 1). The liver of this female hinny contained predominantly horse glucose-6-phosphate dehydrogenase (G6PD), but only donkey mtDNA (Fig. 2). This proved that the animal contained horse chromosomes (G6PD is X linked), and verified the word of the breeder that the animal was, in fact, a hinny. The preponderance of horse G6PD in this hybrid suggests the preferential inactivation of the maternal donkey X chromosome in this animal and is consistent with certain published reports of non-random X-chromosome inactivation in mules and hinnies (ref. 16 and review in ref. 17). It should be noted that a model consisting of an X-linked template for mtDNA synthesis, which is non-randomly inactivated in the hybrid animals, is not a reasonable explanation for the observed maternal inheritance of mtDNA since in this hinny it is the

Maternal inheritance of mammalian mitochondrial DNA

EUKARYOTIC cells contain a class of cytoplasmic DNA molecules which are found only within the mitochondria, where they replicate and are transcribed (for a general review see ref. 1). This mitochondrial DNA (mtDNA) constitutes the 'mitochondrial genome' and carries genetic information essential to mitochondrial function. In view of its extranuclear location, it is not surprising that inheritance of the mitochondrial genome is not governed by the same rules that apply to chromosomal genes. For fungi²⁻⁷ and amphibians⁸ there is evidence that the mitochondrial genome of an individual is derived solely from the maternal parent (in contrast to chromosomal genes, which are inherited biparentally). We present here evidence that the mitochondrial genome is inherited maternally in mammals too.

This problem is not readily approachable by the standard techniques of genetic analysis, because mutations in the mammalian mitochondrial genome have not been identified. We have, therefore, used specific cleavage by a restriction endo-

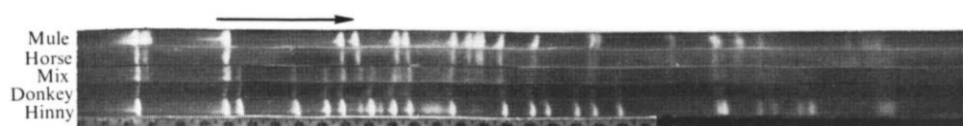


Fig. 1 Polyacrylamide gel electrophoresis of restriction fragments of equine mtDNAs. The arrow indicates the direction of electrophoresis. Reading from the ruler, the samples are: (1) hinny heart; (2) donkey liver; (3) mixture of donkey liver and horse liver; (4) horse liver; (5) mule liver. Analysis of liver mtDNA from the hinny gave a pattern the same as shown here for heart mtDNA. Our preparation of mtDNA will be described in detail elsewhere (manuscript in preparation). Tissue was homogenised to disrupt cells, then nuclei and debris were sedimented in the presence of 0.25 M sucrose at 500g for 10 min. After recentrifuging the supernatant at 500g, the mitochondria were pelleted twice at 10,000g for 10 min. The pellet was suspended in 0.15 M NaCl, 0.05 M EDTA, 0.01 M Tris, pH 7.4, lysed by the addition of 2% sodium dodecyl sulphate (SDS), phenol extracted twice, and then dialysed against SSC. About 20 ml of this mitochondrial nucleic acid preparation was then sedimented into an 18 ml preformed CsCl-ethidium bromide gradient ($\rho = 1.4$ to 1.7; 100 $\mu\text{g ml}^{-1}$ ethidium bromide) poured in a 1×3.5 inch cellulose nitrate tube. The gradient was centrifuged 18–24 h at 22,000 r.p.m. in a SW27 rotor. DNA bands were demonstrated by fluorescence in ultraviolet light, and the bottom (supercoil) band was collected, freed of ethidium bromide by isopropanol extraction, then dialysed against SSC. The mtDNA was pelleted at 33,000 r.p.m. for 10 h in a SW50.1 rotor, the supernatant was discarded, and the mtDNA was dissolved in 0.15 M NaCl, 0.01 M Tris, pH 7.5. Yields from this type of preparation range from about 0.1 to 1.0 μg of supercoiled mtDNA per gram of tissue. To 45 μl of mtDNA solution (containing 10–20 μg DNA) were added 10 μl 0.13 M MgCl_2 , 5 μl 0.02 M β -mercaptoethanol, and 5 μl endonuclease Z (enough enzyme to give a limit digest of 50 μg of SV40 DNA). Digestion was carried out at 37°C for 10 h, solid sucrose was added to 20% and the sample was layered on top of a cylindrical 3.5% polyacrylamide gel (gel composition as in ref. 9 except that agarose was omitted) 7 mm in diameter and about 50 cm in length. Electrophoresis was carried out for about 18 h at 310V, at which time bromophenol blue added to the sample is near the bottom of the gel. Gels were removed gently from the plastic tubes by air pressure into electrode buffer⁹ containing 1 $\mu\text{g ml}^{-1}$ ethidium bromide, and stained at room temperature for 2 h. Gels were placed against a black background, illuminated at a distance of a few inches with a Sylvania type BLB fluorescent black-light bulb and photographed at $f/4.7$ for 60 to 120 s on Polaroid type 55 P/N film. An ultraviolet filter and an orange filter approximating the colour of the DNA-ethidium fluorescence were placed in front of the camera lens. These fragment patterns are interpretable in terms of a single type of mtDNA molecule of approximately 1×10^7 daltons for each of the two equine species. A quantitative analysis and comparison of these patterns will be presented elsewhere.

paternal X which is active. In summary, the evidence presented here demonstrates that the mtDNA of both mules and hinnies is maternally derived. We believe that as little as 5% paternal mtDNA would have been detectable in our experiments; however, we cannot exclude the presence of smaller amounts of paternal mtDNA. It seems reasonable to assume that the experiments look directly at inheritance of the primary nucleotide sequence of the mtDNA, however, we cannot logically exclude the possibility that factors which modify cleavage sites on the mtDNA differ between the two species and are maternally inherited.

Since the mtDNA is maternally inherited in both reciprocal hybrids, it seems reasonable to conclude that mtDNA is maternally inherited in each of these equine species. It would be surprising if this phenomenon were restricted to equine mammals. These experiments support the idea that the mitochond-

drial genome consists of cytoplasmically replicating mtDNA which is not the product of any chromosomal template.

Although it is not surprising that mtDNA is inherited maternally in mammals it could not be predicted *a priori* since the middle piece of the mammalian spermatozoon, containing the mitochondria, usually enters the egg¹⁸. In the mouse, mitochondria from the sperm have been observed to scatter in the cytoplasm of the egg^{19,20}. In the light of these observations at least two mechanisms for maternal inheritance can be proposed: (1) the paternal (spermatozoon) mitochondria may be incapable of replication during development of the fertilised eggs, (2) maternal inheritance may result from a quantitative preponderance of maternal (egg) mitochondria in the zygote. It is amusing to consider some numerical consequences of the second type of mechanism. It has been estimated that a bull sperm contains 72 mitochondria²¹. Mammalian eggs are known to be rich in mitochondria^{18,22}, and if the concentration of mtDNA is similar to that found in the cytoplasm of echinoderm eggs^{23,24}, an equine egg would contain about 10^6 molecules of mtDNA. This would imply that if paternal and maternal mitochondria replicate at equal rates, then only about 10^{-4} of the mtDNA in a mammal would be paternally derived. This would be below the limit of detection of our experiments. Furthermore, the sperm mitochondria could be partitioned into cells during the formation of the blastocyst, before the onset of mitochondrial replication. As a consequence, many clones of cells in the developing embryo may be completely free of paternal mtDNA, even if paternal mitochondria are viable. It therefore seems unnecessary to postulate any replicational defect in the paternal mitochondria to account for the observed maternal inheritance of mtDNA. The possibility still exists that paternal mitochondrial genomes are present in very small numbers in mammalian tissues, and that sufficiently sensitive experiments could detect their presence.

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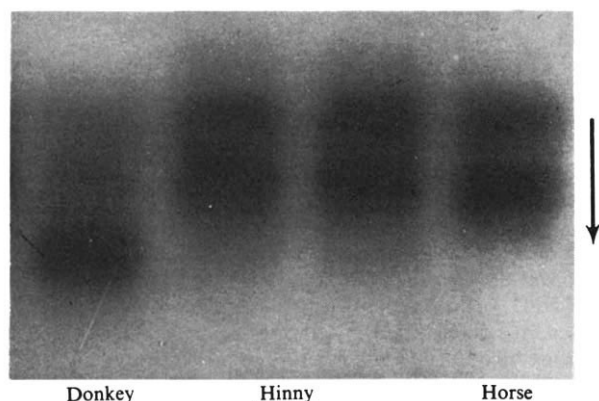


Fig. 2 Starch gel electrophoresis of equine G6PDs. Liver extracts were electrophoresed by the Tris-EDTA-borate method and stained for G6PD activity²⁵. The left channel contains donkey G6PD, the two middle channels are identical samples of female hinny G6PD, and the right hand channel is horse G6PD. An arrow indicates the direction of electrophoresis. It has been suggested²⁶ that the slower moving (minor) components in both horse and donkey G6PD represent degradation products. The hinny samples are clearly predominantly horse G6PD, but appear possibly to contain a trace of the faster moving donkey enzyme.

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T3 x T7 phage crosses leading to recombinant RNA polymerases

THE related virulent bacteriophages T3 and T7 code for RNA polymerases which are specific for promoters located on the DNA of these phages^{1,2}. These single peptide chain polymerases—the products of gene 1 (refs 1 and 3)—are genetically homologous but can be distinguished by their template specificity and their molecular weight. The T3 RNA polymerase transcribes T7 DNA at only about 10% of the rate at which T3 DNA is transcribed, and the T7 RNA polymerase transcribes T3 DNA at about 60% the homologous rate²; and, by direct comparison with the T7 polymerase, the molecular weight of the T3 polymerase was found to be about 5% lower than that of the corresponding T7 protein^{4,5} (whose molecular weight has been estimated at 107,000 (ref. 1), 110,000 (ref. 2), 100,000 (ref. 6), and 108,000 (ref. 4). Since phages T3 and T7 can be crossed⁷, the differences between the T3 and T7 RNA polymerases provide a model system for studies on the correlation between genetic constitution and functional

specificity. Here we report some initial experiments in this direction.

The procedure was to isolate genetic recombinants within gene 1 of T3 and T7, and to analyse the corresponding gene 1 products with regard to template specificity and molecular weight. Since the recombination frequency in crosses between T3 and T7 is very low^{7,8}, parental types carrying two amber mutants were chosen to avoid *am*+ revertants. One parent was T7 *am*H13.*am*H280 (with both *am* markers within gene 1) and the other was T3 *am*H222.*am*H283 (a gene 1-gene 3 double mutant). The exact locations of the mutational sites are shown in Fig. 3. The proportion of *am*+ recombinants among the pro-

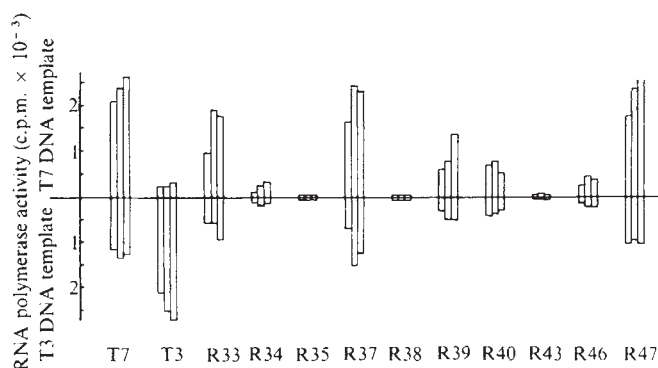


Fig. 1 RNA polymerase activity in cell-free extracts of phage-infected host bacteria, assayed with T3 or T7 template DNA. Exponentially growing cells of *E. coli* B (2×10^8 cells ml⁻¹) in casaminoacids medium⁸ (37°C) were infected with T7, T3 or various genetic recombinants between T3 and T7 (multiplicity: 10 PFU/cell). At 5, 7 and 9 min after infection (first, second and third column, respectively), sample (10 ml) of each culture was poured on ice with chloramphenicol (300 μ g ml⁻¹, final concentration) and centrifuged immediately. The pellets were resuspended in 0.5 ml buffer (0.05 M Tris-Cl, pH 8; 0.01 M 2-mercaptoethanol; 2 mg ml⁻¹ bovine serum albumin) and the cells were disrupted by low intensity ultrasound (Branson sonifier B-12, microtip, 0°C). The extracts were centrifuged and the supernatant fluid was assayed at 37°C for rifampicin-resistant RNA polymerase activity as described by Chamberlin *et al.*¹, except that first, 100 μ l of assay mixture contained 7 μ g of T3 or T7 DNA and second, 20 μ l of cell extract were added to 100 μ l of assay mixture. The RNA polymerase activity is here given as c.p.m. of ¹⁴C-UTP (500 c.p.m. nmol⁻¹) incorporated into acid precipitable material, per assay.

geny of such crosses was $1-4 \times 10^{-5}$; that is, about a thousand times less than in corresponding crosses with homologous phage. From such a T3 x T7 cross we picked ten *am*+ plaques for further analysis.

The first step in this analysis aimed at ascertaining the recombinatorial origin of these *am*+ stocks. For this, two non-selected markers were checked: first, the ability to code for an active S-adenosylmethionine-cleaving enzyme (AdoMet-ase), which depends on the T3-specific *sam*+ gene (ref. 3), and second, the specificity towards T3 and T7 antiserum, determined by the product of gene 17 (serum blocking protein)⁹. (The *sam*+ gene is located at the 'left' end of the genetic map³ and gene 17 lies at about 0.9 genome length, that is, near the 'right' end of the map¹⁰.) The assay techniques for these markers were described elsewhere (refs 11 and 9). All the ten *am*+ stocks were *sam*+ and had the antiserum specificity of T7. Thus, these phages were undoubtedly recombinants between T3 and T7.

These T3 x T7 recombinants were then analysed with regard to the activity and template specificity of the phage RNA polymerase they induce after infection of *Escherichia coli* B. As shown in Fig. 1, all the recombinant RNA poly-

merases either had a preference for T7 DNA or had virtually no measurable *in vitro* activity. The recombinants with no activity at 37° C (R35, R38, R43) were also grown and tested at 25° C, with the same result. Since the phage RNA polymerase is essential for phage growth, as evidenced by the fact that amber mutants in gene 1 cannot grow on *su*⁻ hosts, it must be concluded that the RNA polymerases coded by these recombinants are functionally active *in vivo* but are so unstable that they become inactive in the process of extraction. The recombinant RNA polymerases active *in vitro* were also less stable than the wild type enzymes, as shown by a higher temperature sensitivity.

Next, the recombinant RNA polymerases were characterised with regard to their molecular weights. This was done by polyacrylamide gel electrophoresis, in the presence of SDS, of extracts of host cells infected with phage and supplemented with ³⁵S-methionine, followed by autoradiography of the dried gels. This method (successfully used to characterise the T7-coded intracellular proteins^{8,12}) revealed that the RNA polymerases of three of the tested recombinants had the molecular weight of the T3 RNA polymerase while the others—with one exception—had the molecular weight of the corresponding T7 enzyme (Fig. 2). The one exception, R43, coded for a gene 1 product with a molecular weight intermediate to those of the RNA polymerases of T3 and T7 (Fig. 2). It is especially noteworthy that the RNA polymerases of R33, R39 and R47 showed a preference for the T7 DNA template, although they had the molecular weight of the T3 RNA polymerase.

Our main interpretations of the results are as follows. First, the higher molecular weight of the T7 RNA polymerase is probably not due to a single deletion which occurred in a T3 ancestor or a single insertion in a T7 ancestor. Rather, there seem to be at least two regions

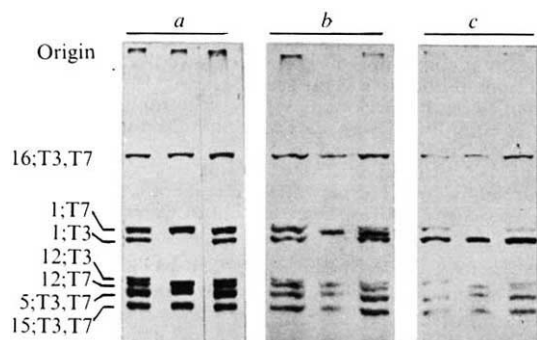


Fig. 2 Comparison of the molecular weight of recombinant RNA polymerases with that of T3 and T7 polymerase. Phage-directed proteins were continuously labelled by adding ³⁵S-methionine (40 μ Ci ml⁻¹, final concentration) together with the phage (multiplicity: 10 PFU per cell) to a sample (0.2 ml) of ultraviolet-irradiated *E. coli* B bacteria (3×10^8 cells ml⁻¹). After 15 min at 37° C the infected cells were collected by centrifugation and resuspended in 20 μ l of buffer (0.15 M Tris-Cl; 1.5 M 2-mercaptoethanol; 5% sodium dodecyl sulphate; 10% glycerol). After 2 h of incubation at 50° C, followed by 2 min at 100° C, the samples were placed on 8% polyacrylamide slab gels and subjected to electrophoresis for 5 h at 150 V. The dried gels were then autoradiographed for 2 d. Further details of the method are as described by Studier¹². In this figure the results for three of the tested recombinants are given: *a* is an example of recombinant RNA polymerases with the molecular weight of the T7 polymerase (here: R34); *b* refers to R43, with a gene 1 product of intermediate molecular weight; and *c* represents the recombinant RNA polymerases with the molecular weight of the T3 enzyme (here: R47). For each recombinant tested, three parallel runs were made: at left is a reference frame which is a mixture of T3-coded and T7-coded proteins (the numbers at left refer to the corresponding genes^{4,6}); the middle column is that of the recombinant proteins and at the right a mixture of T3, T7 and recombinant proteins was applied.

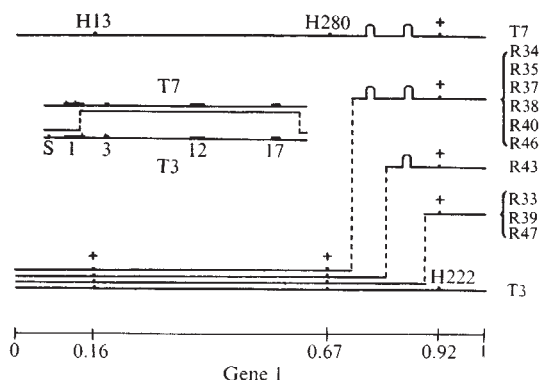


Fig. 3 Scheme of the presumptive genetic constitution of T3-T7 recombinants within gene 1. These recombinants were picked as *am*⁺ plaques after plating the progeny of the cross T7^{am}H13-*am*H280 $\times$ T3^{am}H222-*am*H283 on the *su*⁻ host *E. coli* B. For the crossing procedure see ref. 3. The T7 parent carries two amber markers within gene 1 and the T3 parent is a gene 1-gene 3 double mutant. The position of the *am* markers within gene 1 was determined by estimating the molecular weights of the gene 1 amber peptides with the gel electrophoresis-autoradiography method¹². The inset represents the length of the complete genomes of the parental phages^{3,10} and the probable overall genetic constitution of the recombinants. The specified genes are those for which this constitution has been checked (*S=sam*). The presumed crossover at the right end of the genome is to satisfy the need for identical terminal redundancies¹⁰ at the ends of recombinant phages; alternatively, such a crossover could have taken place to the left of the *sam* gene.

(‘loops’) of about equal size within gene 1 of T7, which have no homologous counterpart in gene 1 of T3. Otherwise, a recombination event leading to a gene 1 of a size intermediate between that of T3 and of T7 would be highly unlikely to occur. These loops (Fig. 3) comprise about 150–200 base pairs each, as judged by the physical length of gene 1, and may correspond to the region of non-homology between T3 and T7, seen at about 0.15 genome length through electron microscopy of heteroduplex T3-T7 DNA (ref. 10). Second, gene 1 polypeptides whose primary structure over a large extent is that of the T3 enzyme but which carry the amino acid sequence encoded by one or both loops of T7 gene 1 are likely to fold into unstable tertiary structures; therefore the *in vitro* inactivity or temperature sensitivity of the recombinant enzymes. Third, the gene 1 segment which imparts the template specificity (either a preference for the DNA of T3 or T7) to the RNA polymerase is located at the ‘right’, that is the carboxyl end of gene 1. Thus, recombination events occurring distal to the loops of T7 gene 1 may lead to recombinant genes coding for RNA polymerases with a molecular weight characteristic of T3 but a template specificity characteristic of T7.

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DNA purified from naked intranuclear particles of human liver infected with hepatitis B virus

DANE *et al.*¹ observed a particle 42 nm in diameter with a double shell and an inner core resembling a viral nucleoid in the sera of some patients with hepatitis B antigenemia. They postulated that this particle was the complete hepatitis B virion. Almeida *et al.*² showed that an internal core particle, 27 nm in diameter, could be released by treating the 42 nm Dane particle with Tween-80 (polysorbate) and that this core particle contained an antigen different from hepatitis B antigen (HBsAg). Almeida³ suggested that these core particles were identical to naked particles, also 27 nm in diameter, observed by electron microscopy in the nuclei of hepatocytes of human livers infected with hepatitis B virus (HBV)^{4,5}. Cores isolated from Dane particles in human sera⁶ seem to be similar in morphology to the 27 nm naked particles visualised in homogenates of HBV-infected human livers⁷. We recently reported methods for isolation and purification of concentrated preparations of naked intranuclear particles of hepatocytes of HBV infected liver⁸. The ultraviolet absorption spectrum of these particles suspended in 0.05 M Tris-HCl, 40 mM KCl, 0.1% β -mercaptoethanol (BME), 10 mM MgCl₂, pH 7.5 buffer showed a peak at 264 nm and a shoulder at 280 nm indicating the presence of both nucleic acid and protein⁸. We now report the isolation of double-stranded DNA from these particles and describe the spectral properties of the isolated DNA.

The naked intranuclear particles of the hepatocytes of an HBV-infected human liver obtained at necropsy from a patient with chronic HBV infection were purified as previously described⁸. The purification procedure entailed the isolation of the liver nuclei, the subsequent release of the naked intranuclear particles from the nuclei by sonication, and the purification of the particles on CsCl density gradients⁸. The purified particles obtained from the CsCl gradients at a density of 1.30–1.31 gm ml⁻¹ (ref. 8), and visualised by electron microscopy⁸, were dialysed against 0.01 M NaCl, 0.01 M PO₄, 10⁻³ M EDTA, 1% BME, pH 7.2 buffer. The particle preparation was made up to a concentration of 1% of sodium dodecyl sulphate (SDS), allowed to stand at room temperature for 10 min, and then extracted four times at room temperature with distilled phenol previously equilibrated with buffer. The aqueous portion was dialysed exhaustively against two changes

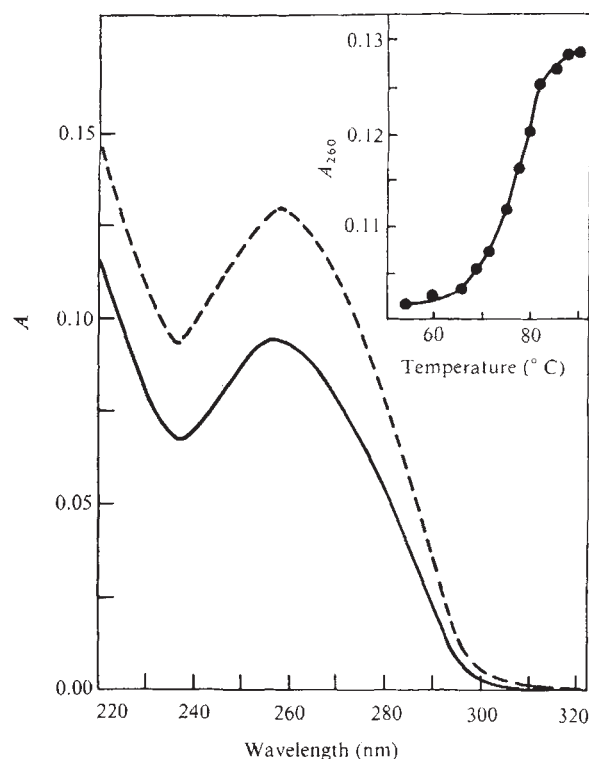


Fig. 1 Ultraviolet absorption spectra of DNA purified from naked intranuclear particles of hepatocytes of HBV-infected human liver. Solid line: native spectrum at 33.6°C; dashed line: denatured spectrum at 87°C. Insert shows the thermal denaturation profile at 260 nm. DNA was dissolved in 0.01 M NaCl, 0.01 M PO₄, pH 7.2 buffer. 100 μ l microcuvettes of 1 cm pathlength contained 0.35 ml DNA solution to preclude meniscus effects on the absorbance values. The microcells were fitted with Teflon stoppers and sealed with RTV-60 compound. Temperature was controlled with a Laude K-2R circulation bath (Brinkmann Instruments) containing 50% ethylene glycol and measured by a platinum probe in a 3 ml sealed quartz cuvette filled with 1 ml buffer in the Beckman Acta V spectrophotometer. Temperature equilibrium was reached within 20 min at each point. The temperature was raised by 5°C increments until 50°C and then by 1–2°C until denaturation was complete. Absorbance values read by digital display at each temperature were corrected to 4°C for the expansion of water¹³.

of 0.01 M NaCl, 0.01 M PO₄, 10⁻³ M EDTA, pH 7.2 buffer and then against four changes of 0.01 M NaCl, 0.01 M PO₄, pH 7.2 buffer at 5°C.

The ultraviolet absorption spectrum of the resulting dialysate suggested a solution of nucleic acid (Fig. 1). The nucleic acid was identified originally as double-stranded DNA by means of the following reactions: (1) a positive diphenylamine⁹ but negative orcinol¹⁰ reaction; (2) immediate increase in ultraviolet (220–290 nm) absorbance (+12% at 260 nm) when material of 0.1 absorbance at 260 nm was treated with pancreatic DNase (20 μ g ml⁻¹; Sigma; double-stranded calf thymus and *Bacillus subtilis* DNAs gave comparable immediate increases in ultraviolet absorbance when treated with DNase under similar conditions, the immediate

Table 1 Properties of DNA purified from naked HBV particles determined by spectral analysis

	Hyperchromic spectra	Denatured spectra	
Mole fraction A-T	0.434 $\pm$ 0.012 (s.d.)	0.374 $\pm$ 0.023 (s.d.)	Average 0.404 $\pm$ 0.036 (s.d.)
Concentration (mol nucleotide l ⁻¹)	1.35 $\times$ 10 ⁻⁵	1.41 $\times$ 10 ⁻⁵	DNA in native state (%), 95.7
Molar extinction at 260 nm (l mol ⁻¹ cm ⁻¹)	6,926	6,631	Average 6,779
Randomness (δ) parameter	0.012	0.124	

See text and legend to Fig. 1 for experimental details. Calculations were based upon data taken at 5 nm intervals between 220 and 290 nm for the hyperchromic spectra and at 240 nm, 250 nm, 260 nm, 270 nm and 280 nm for the denatured spectra using the three term analyses given by Hirschman and Felsenfeld¹⁴. A program written for a Wang 700B computer was used for the calculations.

increase in absorbance representing incomplete digestion of double-stranded DNA) and no change in absorbance when treated with pancreatic RNase ($20 \mu\text{g ml}^{-1}$; Sigma); (3) no reaction when neutralised formaldehyde was added to a concentration of 1.8% (ref. 11); (4) a very slow increase in ultraviolet absorbance of the heat-denatured material treated with pancreatic DNase but no reaction when treated with pancreatic RNase; and (5) a slow increase (single-stranded RNA reacts immediately) in ultraviolet absorbance ($+15\%$ at 260 nm in 4 h) when the heat-denatured material was treated with neutralised formaldehyde (1.8%). Electron microscopic observation¹² of the material showed linear strands consistent with double-stranded DNA.

First estimates from the absorbance at 260 nm indicated that the yield of double-stranded DNA from naked particles purified from 60 g of HBV infected liver was 15 μg . It was considered that the properties of such modest amounts of DNA could well be studied by spectral analysis¹³⁻¹⁵ adapted to microdeterminations. Ultraviolet spectra were recorded with a Beckman Acta V spectrophotometer in the single beam mode using 100 μl microcuvettes and microapertures. DNA of known base composition in 0.01 M NaCl, 0.01 M PO_4 , pH 7.2 buffer also was analysed to determine the accuracy of the micro-methods. The microcells were fitted with Teflon stoppers and sealed with RTV silicone rubber compound (General Electric) for DNA melting experiments^{13,14}. Analysis of DNA of known base composition (*B. subtilis* and calf thymus DNAs, 0.665 and 0.585 mole fraction A-T, respectively) adjusted to 0.1 absorbance at 260 nm at room temperature showed: (1) accurate determination of the blank values of the microcuvettes was imperative; (2) correct assessment of the first temperature of complete denaturation¹³ was critical, especially when deriving data from the denatured spectra, because the small changes in absorbance produced by depurination upon further heating¹³ led to large errors in values of mole fraction A-T; (3) analyses of the denatured spectra gave lower values of A-T mole fraction, that showed greater scatter, than those derived from the hyperchromic spectra; (4) the low initial absorbance values required precise measurement of the temperature at each point because of the relatively greater contribution to the total absorbance values by the corrections for the expansion of water at each temperature; and (5) the microanalyses of DNA of known base composition were accurate to ± 0.040 and ± 0.055 mole fraction A-T using the hyperchromic and denatured spectra, respectively. These values compare well with the macromethods¹⁴. The native spectra were not used in these studies because of the greater inaccuracy of the derived values of mole fraction A-T¹⁴. Only three-term analyses¹⁴ were used to derive data from the spectra.

The ultraviolet absorption spectrum of the purified native DNA showed a peak at 257 nm and a trough at 237 nm (Fig. 1). When heated to 90°C the DNA gave a sharp rise in hyperchromism of 33% at 260 nm (Fig. 1, insert); the melting temperature (T_m) in 0.01 M NaCl, 0.01 M PO_4 , pH 7.2 buffer was 77°C , characteristic of DNA of 0.44 mole fraction A-T (S. Z. Hirschman, unpublished data).

The mole fraction A-T derived from the hyperchromic spectra (Fig. 1) was 0.434 ± 0.012 (standard deviation, s.d.) and from the denatured spectra 0.374 ± 0.023 ; the average value from both spectra was 0.404 ± 0.036 (Table 1). Analysis of the hyperchromic and denatured spectra yielded DNA concentrations of 1.35×10^{-5} and 1.41×10^{-5} mol nucleotide $^{-1}$, respectively, indicating that 95.7% of the DNA was in native (double-stranded) state¹⁴ (Table 1). The average molar extinction coefficient at 260 nm was $6,779 \text{ l mol}^{-1} \text{ cm}^{-1}$ (Table 1). The values of the 'randomness' (δ) parameters^{13,14} were 0.012 and 0.124 for the hyperchromic and denatured spectra, respectively.

Preliminary comparisons of the fraction of all A-T pairs (F_{AT}) against the fraction of all G-C (F_{GC}) pairs melted at each temperature^{11,15} indicated that 60% of the DNA had a mole fraction A-T of 0.48 and 40% had a mole fraction A-T of 0.32.

But because of the small changes in absorbance at each wavelength at each temperature throughout the melting these data will require substantiation by studies with much higher concentrations of DNA.

Lower values of mole fraction A-T derived from the denatured spectra were found also with DNAs of known base composition (see above). The value of mole fraction A-T derived from the hyperchromic spectra agreed with the value obtained from the T_m at 260 nm. The average extinction coefficient at 260 nm of $6,779 \text{ l mol}^{-1} \text{ cm}^{-1}$ compares to the extinction coefficient of $6,710 \text{ l mol}^{-1} \text{ cm}^{-1}$ previously reported for bacterial DNAs of 0.43 mole fraction A-T¹³. The very low values of the 'randomness' (δ) parameter^{13,14} suggested that the DNA did not contain abnormal bases.

Thus, double-stranded DNA of unique base composition has been isolated from the putative HBV nucleoid, the naked intranuclear particle of hepatocytes of human liver infected with HBV. Unfortunately, electron microscopic observation of the DNA showed the strands to be heterogeneous in size with a highest molecular weight of about 1×10^6 . We assume that the DNA strands were broken either by liver nucleases or the sonication to release the particles from the nuclei of the hepatocytes during particle preparations⁸. Various methods of particle preparation are under investigation to allow determination of the true molecular weight of the DNA.

The small HBV nucleoid could accommodate a DNA of about 2×10^6 molecular weight. Other small mammalian DNA-containing viruses, such as parvo¹⁶ and adeno associated viruses (AAV)¹⁷, contain single stranded DNA in the virion and may require a helper virus for replication. The DNA of AAV may be packaged as separate plus and minus strands that can reassociate to a double-stranded form after extraction of the DNA¹⁷. Whether HBV DNA is actually double stranded in the virion may still be open to some question. But this question and the possibility that HBV may require a helper virus for replication can be subjected to experimental investigation now that concentrated naked particle preparations are available⁸.

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Immunological destruction of herpes simplex virus I infected cells

THERE are clearly two levels at which the immune response may intervene to induce protection against viral infection. Neutralising antibodies effectively limit spread of free virus in many situations, thus preventing reinfection. In a number of viral diseases, however, it is often not possible to cure the infection by even high titres of neutralising antibodies^{1,2}. In such situations, recovery from infection may require the intervention of a cell-mediated immune response directed at the infected cell. Selective impairment of the host's immunity, for example, by thymectomy or by antilymphocyte serum, markedly increased mortality of mice infected with ectromelia³, and herpes simplex^{4,5}. Macrophage suppression has had a similar effect⁶. In these cases recovery could be conferred by transfer of immune lymphoid cells (ref. 7 and B.R.Z., and A. C. Allison, unpublished). But the nature of the cells involved, and the mechanisms by which they act remain problematic. Our experiments were undertaken to evaluate the contribution of various types of cells to immune reactivity to cells infected with herpes simplex virus I *in vitro*.

Adult C57Bl/6J mice were infected intraperitoneally with 10⁶ plaque-forming units (PFU) of the HFEM strain of herpes simplex virus I, (HSV), grown in chick embryo fibroblasts, and immune lymphoid cells were collected at various times after immunisation. The ability of these cells to destroy HSV infected Chang human liver cells, was assessed by measuring the release of ⁵¹Cr from labelled HSV-infected Chang cells 16 h after treatment with various immune or normal lymphoid cells. In this system, Chang cells in suspension were infected at an input multiplicity of 10. Twenty hours after infection, when lymphoid cell preparations were added, 80% of the cells exhibited viral specific membrane fluorescence. Infected cells were labelled for 45 min at 37° C with 100 µCi ⁵¹CrO₄ (Amersham) washed three times, and 5 × 10⁴ cells were distributed to plastic test tubes. Lymphoid cells were added at a ratio of 40:1 and the cells were cultured in Eagle's

Table 1 Cytolysis of HSV-infected Chang cells by immune or non-immune mouse lymphoid cells

Target cells	Effector cells	% Specific ⁵¹ Cr release
HSV-infected Chang	Nonimmune spleen cells	0
	Immune spleen cells	4.5
	Nonimmune spleen cells + anti-HSV mouse serum (1/100)	4.5
	Immune peritoneal exudate cells	46.0
	Nonimmune peritoneal exudate cells + normal mouse serum (1/100)	0
	Immune spleen cells + nonimmune peritoneal exudate cells (20:1)	30.0
	Nonimmune peritoneal exudate cells + anti-HSV mouse serum (1/100)	90.0
	Anti-HSV mouse serum (1/100)	2.5
	Nonimmune peritoneal exudate cells + anti-HSV mouse serum (1/100)	7.0
Noninfected Chang	Nonimmune peritoneal exudate cells + anti-HSV mouse serum (1/100)	7.0

Mouse anti-HSV serum was added to 5 × 10⁴ target cells in plastic test tubes (Falcon No. 2054). Effector cells were then added at a ratio of 40:1 in a final volume of 1.0 ml. Samples were prepared in triplicate. Percentage lysis was computed as: (Mean ⁵¹Cr release in test group—mean spontaneous ⁵¹Cr release)/(Mean ⁵¹Cr release after freeze-thawing—mean spontaneous ⁵¹Cr release) × 100

medium containing 6% foetal calf serum. Spontaneous ⁵¹Cr leakage was approximately 15–20%.

Repeated attempts to demonstrate the presence of cytolytic lymphocytes in immune spleens were unsuccessful, even in the presence of anti-HSV antibodies (Table 1). In contrast, peritoneal exudate cells (PEC) from immune donors 6 d after infection were markedly cytotoxic for HSV-infected Chang cells. When peritoneal exudate cells from normal mice were admixed with immune spleen cells (at a ratio of 1:20), a significant degree of cytotoxicity was observed, suggesting that the cytotoxicity might be mediated by nonimmune effector cells. This was confirmed by the demonstration that non-immune peritoneal exudate cells in the presence of heat-inactivated anti-HSV rabbit or murine serum had an even greater degree of cytotoxicity than immune peritoneal cells. Immune sera from mouse, rabbit or man were all effective, as demonstrated in Table 2 and both mouse exudate or human peripheral mononuclear cells could serve as effector cells.

The efficiency of cytolysis of HSV-infected cells by antibody-dependent cell-mediated lysis and complement-mediated lysis is compared in Fig. 1. At high concentrations of rabbit anti-HSV antibody, high degrees of lysis of HSV infected cells were observed in both systems. The efficiency of complement-mediated cytolysis however, diminished markedly upon dilution of antibody, whereas the cytotoxicity mediated by

Table 2 Cytolysis of HSV-infected Chang cells by both mouse peritoneal exudate (PEC) or human peripheral blood mononuclear cells (PBL) in the presence of either rabbit or human immune sera

Effector cells	Serum	% Specific ⁵¹ Cr release
PBL	Nonimmune rabbit 1/100	25
PBL	Rabbit anti-HSV 1/100	83
PBL	Nonimmune human 1/100	31
PBL	Human anti-HSV 1/100	81
PEC	Nonimmune rabbit 1/100	10
PEC	Rabbit anti-HSV 1/100	89
PEC	Nonimmune human 1/100	15
PEC	Human anti-HSV 1/100	81

Nonimmune human serum and peripheral blood were taken from donors with no known previous HSV infection. Immune human serum was taken from a patient with recurrent HSV infections. Human peripheral blood mononuclear cells were prepared by Ficoll-Hypaque gradient centrifugation¹⁶. In all cases the lymphocyte target cell ratio was 40:1.

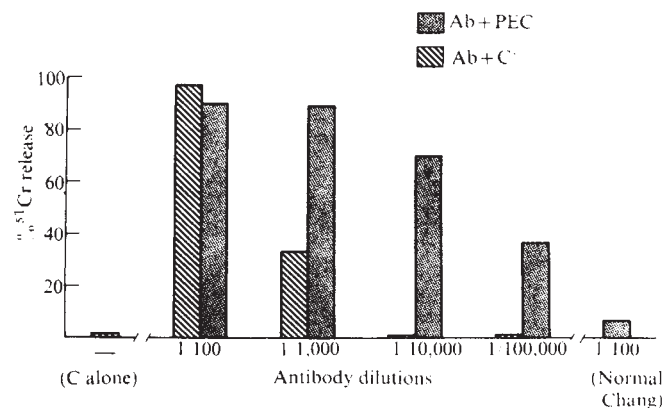


Fig. 1 Comparative efficiency of cytotoxicity of HSV-infected Chang cells by Ab-dependent cell-mediated lysis and complement (C)-mediated lysis. Target cells were treated with dilutions of rabbit anti-HSV serum before the addition of effector cells or complement. The neutralisation titre of the anti-HSV serum was 1:640. Complement was used at a 1:12 dilution of fresh guinea pig serum. The ratio of PEC to target cells was 40:1.

nonimmune peritoneal exudate cells remained high even in the presence of a 1/10,000 antibody dilution. A similarly pronounced effect was observed in the case of murine anti-HSV antibodies obtained early (6 d) after immunisation, in which 52% chromium release was obtained in the presence of normal peritoneal exudate cells with but 3.5% in the presence of guinea pig complement.

These observations confirm the prediction of Allison¹ that antibody-dependent cell-mediated cytotoxicity would be operative in viral immunity, and extend the fundamental studies of Perlmann⁸, MacLennan⁹ and Evans and Alexander¹⁰ to the case of virus-infected cells. Although the precise nature of the effector cell(s) in these systems has not yet been fully elucidated, it clearly does not share characteristics with thymus-derived lymphocytes^{1,9}. Whether it belongs to a subpopulation of B lymphocytes macrophages or is a novel cell type remains unclear, but the effector cells in the mouse have been found to be surface-adherent, poorly phagocytic and dependent on an Fc receptor. Here we confirm that removal of adherent cells from the peritoneal exudate population by culture for 2 h in 60 mm plastic Petri dishes results in a 90% diminution in cytotoxic activity of the cells.

In their studies on the part played by antibodies and cell-mediated reactions on spread of HSV infection *in vitro*, Lodmell *et al.*¹¹ noted that spread of infection had occurred as early as 2 h before the detection of surface HSV antigen by complement-mediated cytotoxicity, and suggested that consequently antibodies were not likely to be useful in limiting the spread of infection. Our studies indicate that antibody-dependent cell-mediated cytolysis of HSV infected cells provides a mechanism orders of magnitude more efficient than complement-mediated cytolysis in terms of antibody required. While extrapolation of the *in vitro* results to the *in vivo* situation would be premature, the data suggest that a collaboration of antibody and the appropriate effector cells could provide a significant degree of restriction of infection. In addition, other cell-mediated mechanisms, such as T-cell dependent cytotoxicity^{12,13} and possibly generation of mediators as a consequence of the inflammatory response^{14,15} may be involved.

Note added in proof: Similar results have recently been obtained independently by Shore, S. L., Nahmias, A. J., Starr, S. E., Wood, T. A., and McFarlin, D. E., *Nature* (in the press).

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Pronounced antiviral activity of human interferon on bovine and porcine cells

INTERFERONS prepared from cells of one species may exhibit some activity on cells of others although the titre on heterologous cells is usually considerably lower than that for homologous cells¹. The one exception to date has been the finding that human fibroblast interferon was far more active on heterologous rabbit cells than on human cells^{2,3}. We report here that human leukocyte and fibroblast interferon preparations exert a pronounced antiviral activity on bovine and porcine cells, and the activity of the human leukocyte interferon is far greater on heterologous bovine cells than on homologous human or monkey cells.

The origins of the interferon preparations and the cells used in their assay are presented in Table 1. Several interferon preparations were prepared in our laboratory and standard techniques were used to eliminate residual inducing virus. All cells were cultivated in Eagle's medium with 10% calf or foetal calf serum. Dilutions of interferon preparations were incubated with cell cultures for 18 h before the test virus was added. Two methods of assay were used. First, inhibition of the cytopathic effect (CPE) of 100 TCID₅₀ of vesicular stomatitis virus (VSV) or encephalomyocarditis (EMC) virus in monolayer cultures of cells in plastic Microtest II Trays (Falcon)⁷. Titres are expressed as the highest two-fold dilution giving approximately 50-75% protection of the cell sheet at a time when virus-infected control cultures showed complete cell sheet destruction. Second, inhibition of virus yield determined 12 or 18 h after addition of VSV or EMC virus at a multiplicity of infection of 1. Titres are expressed as obtained in our laboratory. (One of our units is approximately the equivalent of 1.5-2 human leukocyte MRC reference interferon 69/19 units.)

The antiviral activity of human interferon preparations

Table 1 Interferon preparations

Tissue origin	Interferon inducer	Supplied by
Human leukocyte suspensions	Sendai virus	Dr K. Cantell Dr H. Strander ^{4,5}
Human leukocyte suspensions	Newcastle disease virus (NDV)	Dr T. Kishida ⁶
Human leukocyte suspensions	Sendai virus	Medical Research Council Research standard B 69/19
Human Burkitt cells 'Namalwa line'	NDV	Our laboratory
Human embryonic fibroblasts	Poly(I)·poly(C)	Dr P. Came
Human embryonic fibroblasts	NDV	Our laboratory
Mouse L cells	NDV	Our laboratory
Rabbit RK ₁₃ cells	NDV	Our laboratory
Bovine embryonic skin	NDV	Our laboratory
Porcine (adult) kidney	NDV	Our laboratory
Cells used in assay of interferon		
Species	Cell	Supplied by
Human	Fibroblast WI-38	20 to 28 Established cell line Merieux Laboratories
Monkey	BSC (kidney)	Our laboratory
Cow	Embryonic skin	1 to 15 Our laboratory
Cow	Primary kidney	1 to 6 Merieux Laboratories
Pig	Primary kidney	1 to 6 Merieux Laboratories
Pig	PK ₁₀₆ (kidney)	Established cell line Dr A. Parodi and Dr R. Scherrer
Pig	PK (Kasza)	Established cell line Flow Laboratories

Table 2 Antiviral activity of human interferon preparations on human, monkey, bovine and porcine cells

Origin of interferon preparation	Species cell type	Titre* of interferon on cells challenged with VSV						
		Human	Monkey	Bovine		Porcine		
		WI-38 fibroblast	BSC	Embryonic skin	Adult primary kidney	Adult primary kidney	PK ₁₀₆	PK _(Kauza)
Leukocyte suspensions	Cantell	640,000	640,000	25,000,000	51,000,000	1,200,000	640,000	—
		640,000	1,200,000	25,000,000	12,000,000	—	640,000	640,000
	Kishida	4,000	4,000	32,000	120,000	25,000	3,200	—
		4,000	8,000	120,000	120,000	—	12,000	6,400
	MRC reference 69/19	3,200	3,200	51,200	—	—	—	—
		2,400	2,400	64,000	—	3,200	2,400	1,600
Burkitt cell line 'Namalwa'	Our laboratory	—	1,280	10,240	—	—	—	—
Embryonic fibroblast	Came	6,400	12,800	3,200	—	—	—	—
		—	19,200	—	—	400	1,200	600
		—	12,800	6,400	—	—	—	—
	Our laboratory	—	320	160	—	—	—	—
Heterologous Mouse L cells (titre 1:1,600 on mouse L cells)		—	—	< 20	—	—	< 20	—
Rabbit RK ₁₃ cells (titre 1:3,200 on RK ₁₃ cells)		—	—	< 20	—	—	< 20	—
Bovine embryonic skin		40	—	6,400	—	—	—	—
Porcine kidney		< 10	—	—	—	1:160	—	—

* Titre is given as the reciprocal. All titres on the same line represent the values obtained in simultaneous assays on a given day.
—, Not tested.

on human, monkey, bovine and porcine cells using VSV as the challenge virus is presented in Table 2 and the salient points may be summarised as follows:

Three different human interferon preparations derived from leukocyte suspensions (including the MRC standard reference interferon 69/19, a partially purified interferon (Kishida) and a potent preparation (Cantell) used in the treatment of patients³) all protect bovine cells to a far greater extent than they protect either human fibroblasts or BSC cells. For example, the Cantell preparation had a titre of $1:64 \times 10^4$ on human fibroblasts or monkey kidney cells and a titre of $1:25 \times 10^6$ on bovine embryonic cells.

Human leukocyte interferon preparations protect porcine kidney cells to the same degree as human fibroblasts or BSC cells.

Interferon prepared from a continuous Burkitt cell line behaves as an interferon derived from normal leukocyte suspensions and protects bovine cells to a greater extent than BSC cells.

Human embryonic fibroblast interferon (induced either by poly(I), poly(C) or NDV—Table 1) crosses on bovine and porcine cells. In contrast to leukocyte interferon, however, the antiviral titre of fibroblast interferon was slightly less on bovine cells and considerably less on porcine cells than on human or monkey cells.

Although human interferon preparations exhibited antiviral activity on bovine and porcine cells, these cells are not protected by mouse or rabbit cell culture interferon preparations.

Bovine embryonic skin interferon exhibited only slight antiviral activity, and porcine kidney interferon exhibited no detectable activity on human fibroblasts.

The pronounced antiviral activity of human leukocyte interferon on bovine and porcine cells and the less pronounced activity of human fibroblast interferon on these cells were also observed when EMC virus was used as a challenge virus instead of VSV. Likewise, the pronounced

antiviral activity of the human leukocyte reference interferon on bovine cells was observed when assayed by inhibition of VSV or EMC virus yield rather than by inhibition of CPE.

A number of tests were undertaken using the MRC reference interferon 69/19 to determine the characteristics of the factor responsible for protection of bovine and porcine cells. As can be seen in Table 3, this factor fulfilled the usual criteria for acceptance of a viral inhibitor as an interferon¹. Furthermore, a rabbit antihuman leukocyte interferon serum neutralised (to a titre of 1:800) 4 units of the human leukocyte reference interferon 69/19 as determined in simultaneous assays on monkey, bovine and porcine cells.

Table 3 Characteristics of human leukocyte reference interferon 69/19 as tested on monkey (BSC) bovine embryo (BE) and porcine kidney (PK) cells

Test	Activity on cells		
	BSC	BE	PK ₁₀₆
Washing ($\times 2$) of cell sheet before the addition of challenge virus	++	++	++
Requires several hours incubation on cells for establishment of antiviral activity	++	++	++
Pretreatment of cells for 1 h with 5 μ g actinomycin D	+	0	0
pH 2 for 24 h	++	++	++
Dialysis for 24 h	++	++	++
Supernate after centrifugation 100,000g 1 h	++	++	++
Heating at 60° C for 1 h	+	+	+
Trypsin 500 μ g for 1 h 37° C	0	0	0
DNAase or RNAase 100 μ g 1 h 37° C	++	++	++

VSV used as a challenge virus in these tests. ++, Activity undiminished by treatment; +, approximately 50% loss of activity; ±, approximately 50–90% loss of activity; 0 = > 95% loss of activity.

The finding that human leukocyte interferon exhibited a far greater activity on bovine and porcine cells than human fibroblast interferon of comparable titre is in accord with previous work suggesting that these two interferons differ in their biological^{2,3} as well as in their physicochemical properties. (V. Edy, A. Billiau, M. Joniau, and P. De Somer, unpublished and W. E. Stewart II, P. De Somer and E. De Clercq, unpublished.)

The finding that human leukocyte interferon has such a high titre on bovine cells (that is, Cantell's human leukocyte interferon had a titre of $1:25 \times 10^6$ on bovine cells) may be of value in veterinary medicine. Thus, it may prove simpler to prepare human leukocyte interferon (perhaps from a leukocyte cell line) than to prepare bovine interferon preparations of comparable titre. Furthermore, since bovine cells are highly sensitive to human leukocyte interferon, they may prove useful for the detection of small amounts of this interferon.

It has been suggested⁴ that cells contain specific interferon receptors that may be responsible for the usual species specificity of interferon. If such receptors exist, our results pose the question whether bovine and porcine cells have receptors for both homologous and human interferon or whether these cells share a common interferon receptor.

At least three hypotheses may be suggested to explain why human leukocyte interferon exerts a greater activity on bovine cells than on homologous cells. First, fewer molecules of human leukocyte interferon may be needed to induce antiviral activity in bovine cells than in human cells. Second, there are more interferon receptors on bovine cells than on human cells. Therefore, at limiting dilutions of interferon there is a greater chance of inducing antiviral activity in bovine cells than in human cells. Third, an inhibitor of human leukocyte interferon activity is present in human cells but not in bovine cells.

The explanation of such high activity of interferon on unrelated heterologous cells should prove relevant to the understanding of the nature of the interaction of interferon with cells and the mechanism of the usually observed limited activity of interferon on heterologous cells.

We thank Dr Cantell for supplying rabbit anti-human leukocyte interferon serum, Drs Cantell, Strander, Kishida and Came for human leukocyte and fibroblast interferons, and Dr S. G. Anderson who made available to us the MRC reference interferon. We also thank Drs Parodi and Scherrer for giving us the porcine kidney cell line and Dr Strander for the Burkitt cell line 'Namalwa'.

Note added in proof: Since submission of this article, we have shown that the human leukocyte reference interferon 69/19 and the Cantell human leukocyte interferon also exhibit a far greater antiviral activity on two other bovine cells, embryonic lung (passage six) and embryonic kidney (passage three to five) than on homologous human fibroblasts or monkey kidney cells.

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Reactivation of immunocompetence in spleen cells of aged mice

REACTIVITY in both humoral and cellular immune reactions has been shown to decline with age¹⁻⁴, but the reasons have not yet been critically established. It could be attributed, at least in part, to thymus involution, since the most prominent reduction in reactivity is shown in thymus-dependent responses⁵. The question is, therefore, whether decreasing immune reactivity results from a decrease in the stem cell pool, or whether it is due to a failure in one of the processes of cell maturation which are affected by the thymus.

This study was designed to distinguish between these two possibilities. We used the graft-versus-host (GvH) response as the experimental model, since a decrease in the potential to elicit this response during ageing has been reported⁶. Thus, spleen cells originating in old mice, when injected into allogeneic newborn recipients, produced splenomegaly reactions at a lower level than cells of young mice. To ascertain that the failure to cause splenomegaly

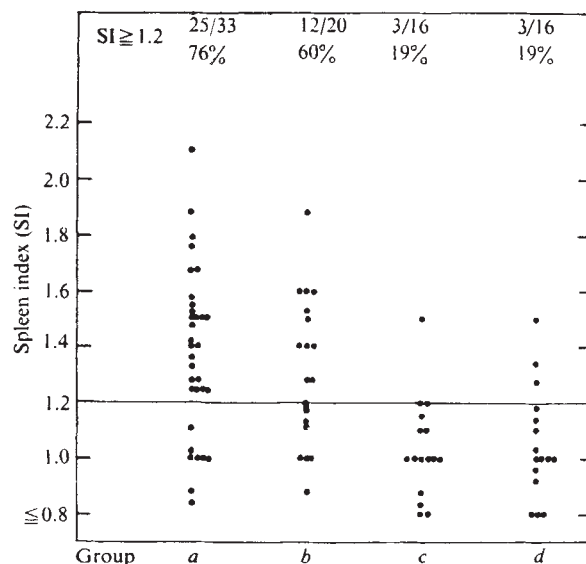


Fig. 1 *In vitro* GvH response by spleen cells of aged mice. Index of newborn BALB/c spleens following incubation with 5×10^5 spleen cells of (C3H/eb $\times$ C57Bl)F₁ mice at different ages. Each point represents the results of one individual culture. Group a, 3 month old mice; b, 12-15 month old mice; c, 23-26 month old mice; d, 32-33 month old mice.

does not result from different homing patterns of the cells introduced to the newborn test animal, we used the *in vitro* technique for the GvH response⁷. We first attempted to establish at which age the reduction in activity is most apparent in the mice used in this study. Thus, (C3H/eb $\times$ C57Bl)F₁ mice were tested at four different ages: 2-3, 12-15, 23-26 and 32-33 months. 5×10^5 spleen cells of each of these groups were added to explants of newborn BALB/c spleens. The relative enlargement of these newborn spleen fragments as compared with fragments cultured with syngeneic spleen cells was recorded after 4 d, as previously described⁷. As shown in Fig. 1, a decrease in the ability of spleen cells to elicit a splenomegaly reaction *in vitro* is manifested with advancing age. The 2-3 month old mice (Group a) elicited the highest incidence of response. High incidence of response was also

obtained in the group of 12–14 month old mice (Group *b*). Cells of the 23–26 (Group *c*) however, and those of the 32–33 month group (Group *d*) manifested a limited reactivity. These *in vitro* results eliminate the possibility that different homing mechanisms account for the failure to produce a response, since the cells were applied directly to the target organ, the newborn spleen. So a reduction in the immune potential of the cells from the aged mice is suggested.

This was further substantiated by increasing the cell doses used to elicit the response. The activity of 10^6 spleen cells from 32–33 month-old mice was tested in comparison with 5×10^6 cells. As seen in Fig. 2, group *a*, the larger dose of cells did lead to a higher incidence of response, 75% as compared to 43%, similar to a higher reactivity obtained upon increase of dose of cells from young mice (Group *b*) (94% as compared to 68%, respectively). Yet, even at that higher dose of cells, the response obtained was relatively low compared to that of the young. These

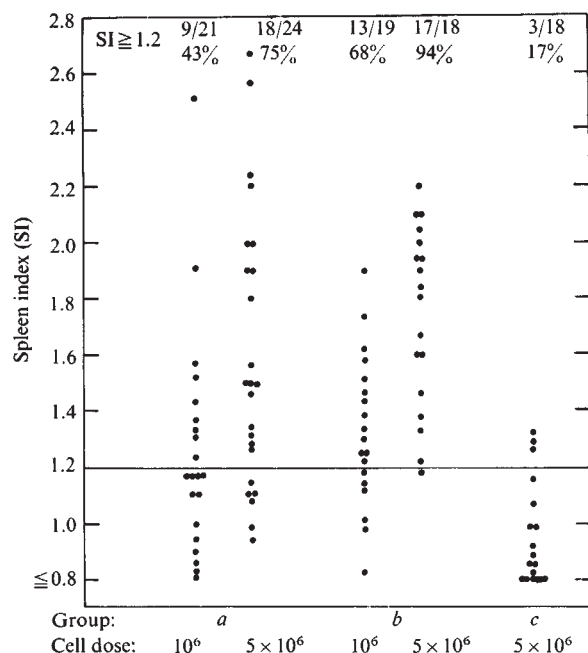


Fig. 2 *In vitro* GvH response by spleen cells of aged mice. Index of newborn BALB/c spleens following incubation with 10^6 or 5×10^6 spleen cells from mice at different ages. Each point represents the results of one individual culture. Group *a*, 32–33 month old (C3H/eb $\times$ C57Bl) F_1 mice; *b*, 3 month old (C3H/eb $\times$ C57Bl) F_1 mice; *c*, 3 month old BALB/c mice.

results could be attributed to a decrease in number of progenitor cells, or to a failure of maturation of progenitors to immune reactive cells as a result of thymus involution. To test this, we planned to apply thymus extract (THF)⁸, since it has been shown to cause cells to become reactive in a GvH response⁹. The *in vitro* GvH experiment was thus repeated, employing spleen cells of 23–26 months, at a dose of 5×10^5 cells, and THF was added to the culture medium, at a concentration of 2% for the entire period of culture¹⁰. Cultures without THF were set up in parallel. Similarly, cells of 2–3 months old mice were tested with and without THF. As shown in Fig. 3, THF enhanced the response elicited by cells from aged donors (Group *a*), whereas it did not lead to any change in reactivity of the cells from young animals (Group *b*).

These results cannot be explained by a deficit in progenitor cells, since no cells were supplied to the system from any external source. We suggest that the decline in the ability to produce a GvH response during ageing stems

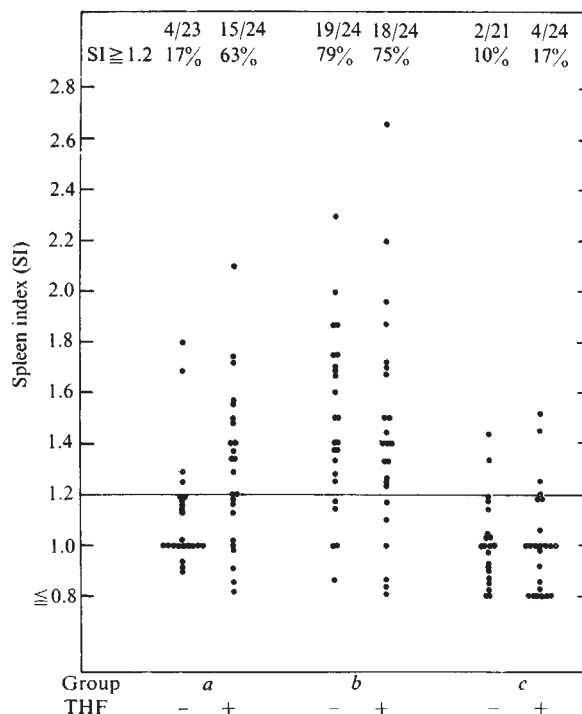


Fig. 3 *In vitro* effect of THF on the ability of spleen cells of aged mice to elicit a GvH response. Index of newborn BALB/c spleens following incubation with 5×10^5 spleen cells from mice at different ages. Each point represents the results of one individual culture. Group *a*, 23–26 month old (C3H/eb $\times$ C57Bl) F_1 mice; *b*, 3 month old (C3H/eb $\times$ C57Bl) F_1 mice; *c*, 3 month old BALB/c mice.

from inadequate activity of thymus hormone, resulting in a failure of maturation.

In earlier studies^{11,12} grafting of young thymus tissue into aged mice had no effect on the response. This may be due to the hormonal activity of the grafts being depressed by central control mechanisms¹³ of the aged recipients.

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Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis

THE cytotoxic activity^{1,2} of immune thymus-derived lymphocytes (T cells) for ⁵¹Cr-labelled fibroblasts, or macrophages infected with lymphocytic choriomeningitis (LCM) virus is restricted by the H-2 gene complex^{3,4}. Specific lysis of LCM-infected monolayer cultures occurs only when targets and overlying, sensitised T cells share at least one set of H-2 antigenic specificities.

Operationally, this restriction may reflect one of two quite distinct mechanisms^{1,2}. First, H-2 compatibility is essential for sufficiently close association³ between lymphocyte and target cell for lysis to occur. This intimacy model implies either that the H-2 gene complex specifies a product, or products, involved in self-recognition, or that there is some mutual interaction between H-2 antigens. Such a process would be additional to recognition of viral antigen by the T cell receptor. The alternative possibility is that infection with LCM virus modifies self components in a way recognised only within a H-2 compatible system. Altered self may be thought of as changes in H-2 antigens (or in structures coded for in the H-2 region) induced by the process of virus synthesis, or as some complex of viral and H-2 antigens.

Lymphocytes from F₁ immune mice lyse virus-infected targets of parent H-2 type as effectively as do syngeneic T cells¹. If the intimacy model is correct, therefore, F₁ mice need possess only one clone of sensitised T cells, recognising viral antigen by a specific receptor and like H-2 antigen, of either parent type, in some non-immunological way (Fig. 1). The altered self hypothesis, however, requires the presence of at least two clones of T cells, each reactive to modified H-2 of one parent type.

These possibilities have been examined using the following experimental system. When LCM-immune T cells are injected into immunosuppressed⁵, virus-infected recipients they home

Table 2 Evidence that sensitised T cells are of donor origin

Donor	Recipient	Treatment	% ⁵¹ Cr release from L cells Infected	% ⁵¹ Cr release from L cells Normal
CBA/H × C57Bl F ₁	AKR	Anti-θ + C	22.3 ± 1.2	21.4 ± 2.5
H-2 ^{k/b} , θC3H	H-2 ^k , θAKR	N ascitic + C	89.1 ± 1.8	23.3 ± 1.2

The protocol is the same as in Table 1, except that a proportion of lymphoid cells were treated³ with AKR anti-θ (C3H) ascitic fluid and rabbit complement or normal AKR ascitic fluid and complement.

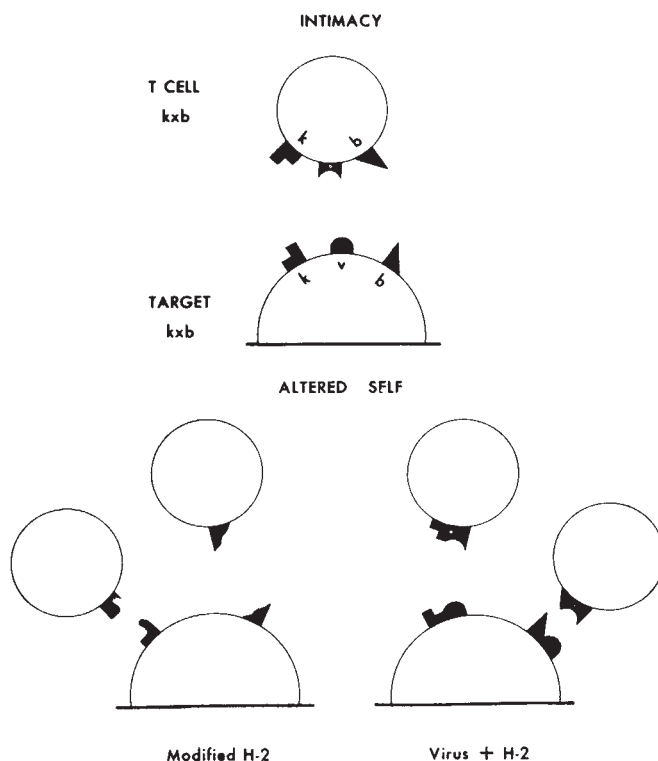


Fig. 1 Capacity of sensitised F₁ (H-2^{k/b}) T cells to interact only with histocompatible virus-infected target cells may be considered to reflect any one of the models shown. The intimacy concept proposes a single immunologically specific T cell receptor for viral (v) antigen, additional to a requirement for physiological interaction coded for by the H-2 gene complex (mutuality between either H-2^k or H-2^b). The two models proposed for altered self postulate that, in each case, there are at least two T cell populations with receptors of different immunological specificities recognising modified H-2, or virus + H-2 of either parent type.

equally well to lymphoid tissue of syngeneic or allogeneic hosts, but continue to multiply only in the syngeneic system⁶. Further replication is apparently not triggered by free virus, but is dependent on thymus-derived lymphocytes being exposed to histocompatible, virus-infected target cells.

Continued proliferation of F₁ (H-2^{k/b}) immune spleen cells with capacity to lyse H-2^k LCM-infected monolayers occurs only in recipients with H-2^k antigenic specificities, and not in those possessing H-2^d (Table 1). These sensitised T cells are of donor origin, as lymphocytes recovered from H-2 compatible AKR recipients adoptively immunised with CBA/H × C57Bl spleen cells were shown to bear θ C3H (Table 2). The intimacy hypothesis, therefore, is probably not correct, as the postulated single clone of F₁ lymphocytes (Fig. 1) should continue to proliferate equally well in recipients of either parent strain. The results cannot, however, exclude this model totally, the reservation being that there may be allelic exclusion in the F₁ of this hypothetical self-recognition product. The simplest

Table 1 Cytotoxic activity of donor T cells in LCM-infected irradiated recipients*

Donor cells*	Recipient strain	H-2 type	% ⁵¹ Cr release† from L cells (H-2 ^k) Infected	% ⁵¹ Cr release† from L cells (H-2 ^k) Normal
Experiment 1				
Immune	CBA/H × BALB/c	k/d	51.2 ± 0.5	12.5 ± 1.9
	C57Bl × BALB/c	b/d	17.3 ± 0.1	13.9 ± 1.3
	BALB/c	d	16.1 ± 1.8	13.0 ± 1.3
Normal	BALB/c	d	14.4 ± 1.9	11.7 ± 1.1
Experiment 2				
Immune	CBA/H	k	83.7 ± 5.4	19.0 ± 1.4
	C57Bl	b	23.3 ± 1.3	19.1 ± 1.0
Normal	C57Bl	b	20.3 ± 1.2	18.5 ± 1.7

*Donor CBA/H × C57Bl F₁ were injected intracerebrally with 300 LD₅₀ of WE3 LCM virus and killed when clinically affected 7 d later. Recipients were irradiated (850 r.) 24 h before i.v. injection of 10⁶ LD₅₀ of WE3 LCM virus, and inoculated i.v. with 5.0 × 10⁷ spleen and lymph node cells 6 h later.

†% ⁵¹Cr release above normal values is a measure of activity of H-2 compatible sensitised T cells^{1,4}. Spleen cells from LCM-immune mice caused % ⁵¹Cr release of 46.6 ± 0.9% from infected L cells and 15.2 ± 1.3% from normal L cells (experiment 1).

explanation of our results is, however, that there are sensitised T cells of at least two specificities in LCM-infected H-2^{k/b} mice, each recognising a complex of virus + H-2 (or modified H-2) of one parent type. Recirculating T cells may function essentially to survey the integrity of transplantation antigens, or structures coded for by the H-2 gene complex. Recognition of cell surface changes due to virus infection, chemical modification⁷ or genetic difference (alloantigens) may then be accommodated within the same model.

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Immunodeficiency and runting syndrome in rats from congenital pyridoxine deficiency

CONGENITAL pyridoxine deficiency is teratogenic in the rat, producing digital defects, cleft palate, omphalocele, micrognathia, exencephaly and hypoplasia of the spleen and thymus¹. Surviving offspring may develop convulsions²⁻⁴, presumably because of a delay in the development of the γ -aminobutyrate shunt in the brain⁵. Congenital pyridoxine deficiency also causes cleft palate in the mouse⁶. I have now found that congenital pyridoxine deficiency has an adverse effect on the immunological function of surviving pups.

I used six dietary groups: one with congenital pyridoxine deficiency (group *a*) and five control groups (*b-f*). Virgin female Sprague-Dawley rats were placed with males in the evening. The morning after mating, when copulatory plugs or sperm could be found, was counted as day 0. To obtain group *a*, we gave pregnant rats pyridoxine-deficient diet (Nutritional Biochemicals) with 0.1 mg ml⁻¹ 4-deoxypyridoxine (4-D), an antagonist of pyridoxine in their drinking water from day 4, 5 or 6 to day 21 of gestation, when they were returned to a regular diet and tap water. All these rats developed dermatitis and other clinical signs of pyridoxine deficiency which disappeared within a few days after resumption of the regular diet. Convulsions were not observed among the newborn offspring, possibly because the dams were given the regular diet before delivery. To obtain group *b*, I gave pregnant rats pyridoxine-deficient diet with antagonist in the water and supplemental pyridoxine (1.0 mg ml⁻¹) in the drinking water from day 5 to day 21 of gestation. Group *c* received the regular diet and tap water throughout gestation. Group *d* was given 8 g of

the regular diet each day, which constituted a dietary restriction severe enough to limit maternal and foetal growth without causing anomalies in Sprague-Dawley rats⁷. Group *e* was given 8 g per day of pyridoxine-deficient diet with antagonist and excess pyridoxine in the drinking water because of the possibility that a restricted intake of the deficient diet might lead to some deficiency other than pyridoxine. Group *f* was given equal molar concentrations of antagonist and pyridoxine in the drinking water.

Mean weights for the litters for the first 4 weeks of life are in Table 1. Rats in *d* and *e* were small at birth while those in all control groups grew rapidly for the first 4 weeks. No control pup developed runting. Rats in litter 1 of group *a* grew slowly. A male rat in litter 1 and a male rat and three females in litter 2 developed a runting syndrome.

Rats were weaned at 28 d of age, individually identified with ear tags and separated in cages according to sex. Rats in group *a* which did not develop a runting syndrome grew vigorously and all rats in the five control groups grew vigorously. Weights are shown in Table 2 for the second month of life for male rats in group *a* and group *c*. Two females in litter 2 with the runting syndrome died between 32 and 39 d of age. A third female runt in that litter was killed at 60 d of age when its weight was 43 g.

As shown in the Tables, there was considerable variation from litter to litter with respect to the frequency of runting and the rate of growth. In some litters all rats grew normally and there was no runting.

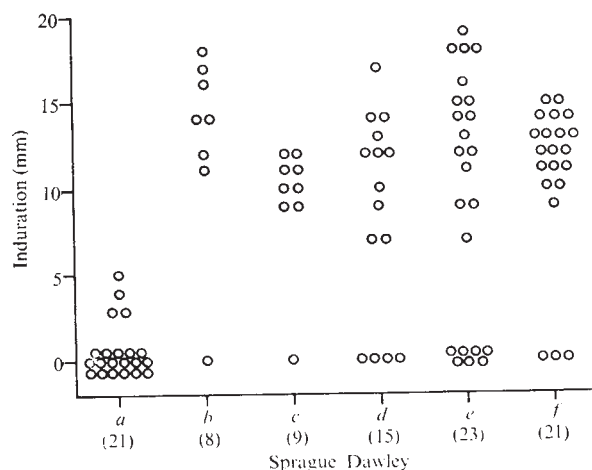


Fig. 1 Results of tuberculin test at 8 weeks of age.

I next bred 12 female Sprague-Dawley rats and gave them the diet for group *b* from day 5 to day 21 of gestation. Two rats were not pregnant (17%). The average maternal weight gain was 155 g. The 10 pregnant rats gave birth to 114 live and one dead offspring. The average birth weight was 7.0 g. No anomalies were recognised among the offspring, and none of them developed a runting syndrome. All survived to 6 weeks of age when immunisations were begun.

Thirty-eight rats were bred and given dietary treatment *a* from day 5 to day 21 of gestation. Five rats were not pregnant (13%). Two pregnant rats died in late gestation, and two died on the day of delivery. The average maternal weight gain was 20 g. Of the 429 pups born, 207 died or were eaten on the first day. Average birth weight was 4.8 g. Ninety-four other pups died before 6 weeks of age, leaving 128 alive (30%). Eight of the survivors had developed a runting syndrome by 6 weeks of age. Several rats with a runting syndrome were killed and examined histologically.

Table 1 Average weights in the first month of life of rats treated with congenital pyridoxine deficiency (group *a*) or with five control diets (groups *b-f*)

Age (d)	Group <i>a</i>				Group <i>b</i> (2 Litters)		Group <i>c</i> (3 Litters)		Group <i>d</i> (3 Litters)		Group <i>e</i> (3 Litters)		Group <i>f</i> (2 Litters)	
	Litter 1		Litter 2		Litter 3		Litter 4							
	<i>n</i>	<i>x</i>	<i>n</i>	<i>x</i>	<i>n</i>	<i>x</i>	<i>n</i>	<i>x</i>	<i>n</i>	<i>x</i>	<i>n</i>	<i>x</i>	<i>n</i>	<i>x</i>
1														
2	12	5.0	14	4.6			11	4.9						
3									35	5.3			21	4.8
4							12	7.9	12	8.7				
5							11	9.1	11	10.8			27	9.3
6	12	5.0	14	8.1										
7					5	13.7								
8									35	9.9			21	12.4
10	10	6.8					11	13.6	11	16.5				
11							12	17.6	12	18.7				
13	8	10.0	14	15.1			11	17.0	11	21			27	19.3
14														
15					5	29								
16	6	13.0							34	19.3			21	23
17														
19							11	20	11	24				
20														
21							12	29	12	25				
22														
23														
24	6	20												
25														

n, Number of pups.*x*, Mean weight in grams.

There were severe atrophic changes in the thymus, spleen and lymph nodes.

To examine the capacity for delayed hypersensitivity, healthy rats with no signs of runting syndrome in dietary groups *a-e* were immunised at 6 weeks of age with 0.05 ml of killed BCG bacilli 3 mg ml⁻¹ in complete Freund's adjuvant. Group *f* was immunised with 8 mg ml⁻¹ *Mycobacterium tuberculosis* in heavy mineral oil. Two weeks later animals were tested intradermally in a shaved flank with 250 tuberculin units of purified protein derivative (Connaught Laboratories). Diameter of induration was recorded 24 h later. Results are shown in Fig. 1. By median tests, the tuberculin reactions of the control groups did not differ significantly from each other and the skin test reactions of group *a* were significantly smaller than for the five control groups.

I next investigated the effect of congenital pyridoxine deficiency on 25 Lewis rats in group *a* and 10 Lewis rats in group *c*. Healthy non-runted rats of both sexes were im-

munised at 6 weeks of age with 0.05 ml killed *M. tuberculosis* (8 mg ml⁻¹ in heavy mineral oil) in one hind footpad and 0.05 ml complete Freund's adjuvant in the other hind footpad. Tuberculin tests were done 2 weeks later. The average diameter of induration for the 10 rats in group *c* was 7.3 mm and only one rat had no induration. Fifteen of 25 rats in group *a* had no tuberculin reaction and the average diameter of the reaction was 2.2 mm. The differences were statistically significant. Work with Lewis rats was abandoned because of their low fertility.

To study antibody responses, healthy male non-runted Sprague-Dawley rats in all six dietary groups were immunised intravenously with 10⁸ sheep red blood cells at 6 weeks of age. These animals had not been used in the tests for delayed hypersensitivity mentioned above. Haemagglutination titres were determined at weekly intervals for 4 weeks on four rats in group *a* and 24 rats among the five control groups. Four weeks after primary immunisation, all rats were given a second intravenous immunisation with

Table 2 Weights from 31 to 60 d of age of male non-runted rats whose dams were pyridoxine-deficient during gestation (group *a*) or were fed a normal diet (group *c*)

Age (d)	Litter 1 Weight (g)	Group <i>a</i>		Litter 3 <i>x</i> ± s.d.	Litter 4 <i>x</i> ± s.d.	Group <i>c</i> (3 Litters)	
		<i>n</i>	<i>x</i> ± s.d.			<i>n</i>	<i>x</i> ± s.d.
31							
32		6*	60 ± 7			4	84 ± 9
33							
35				3	125 ± 12	4	101 ± 10
37	71†					6	91 ± 9
39		6	113 ± 5			12	129 ± 12
41				3	162 ± 12	4	137 ± 13
42							
44	112						
45						12	166 ± 10
46		6	155 ± 8			6	130 ± 11
48				3	205 ± 15	4	170 ± 18
49						8	198 ± 9
52						4	197 ± 9
53		6	190 ± 12				
54				3	216 ± 18	4	210 ± 18
55							
56		6	199 ± 10				
60							

*A male runt weighed 37 g on day 32, 36 g on day 39, 33 g on day 46 and died on day 67.

†A male runt weighed 36 g on day 44, the day of death.

sheep red blood cells and haemagglutination titres were then determined at weekly intervals for 4 weeks. Antibody responses in group *a* fell within the range of those of the control groups after both immunisations. A similar result was obtained after primary and secondary intravenous immunisation with *Escherichia coli* of 4 male Sprague-Dawley rats of group *a* and 26 male rats of the five control groups. Although the groups were small, the results were sufficient to show that congenital pyridoxine deficiency did not cause an obvious deficiency in primary or secondary antibody responses to these two antigens.

At 8 weeks of age, when the congenitally pyridoxine-deficient rats exhibited a defect in delayed hypersensitivity, routine histological examination showed that the thymus, spleen and lymph nodes were in normal condition.

These results demonstrate that congenital pyridoxine deficiency produces a defect in the capacity for expression of delayed hypersensitivity in 8-week-old rats. The normal expression of delayed hypersensitivity in the five control groups excludes deficiency of other dietary factors or maternal protein-calorie malnutrition as causative factors. How long the immunodeficiency persists remains to be determined.

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B-cell suppression of delayed hypersensitivity reactions

DURING certain normal immunisation schedules, suppressor cells are generated and act in suppressing the expression of delayed hypersensitivity reactions^{1,2}. The suppression is immunologically specific and is dependent on living cells. We have suggested that the suppressor cells were B cells because they are depleted when 300 mg kg⁻¹ cyclophosphamide (CY) is given 3 d before sensitisation³⁻⁵.

The most direct method of determining, with greater certainty, which lymphocyte subpopulation is responsible for the suppression of the expression of delayed hypersensitivity reactions is by using cell separation techniques. Antibody coated immunoabsorbent (plastic bead) columns were therefore used to separate T and B lymphocytes.

We used the same *in vivo* models as previously described for the assay of suppressor cells¹. Briefly, Hartley strain guinea pigs, treated with 300 mg kg⁻¹ CY intraperitoneally (LD₁₀₋₂₀) 3 d before immunisation with 1 µg ovalbumin (OA) in Freund's incomplete adjuvant (FIA) given sub-

cutaneously in the footpads, served as cell recipients 8 d later. They were then potentially susceptible to the effect of suppressor cells which they were lacking. In other experiments normal recipients were used. Thrice washed pooled spleen cells (3 × 10⁸ viable as determined by 0.2% trypan blue exclusion) were obtained from animals sensitised with 1 µg OA in FIA subcutaneously 8 d previously. These cells were injected intravenously into the CY treated recipients which were skin tested, 1 h later, by an intradermal injection of 100 µg OA into the shaved flank. Skin test reactivity was assessed as described previously⁶. Spleen cells from the same pool were passed down plastic bead (Degalan V26) columns which had been coated with specifically purified rabbit anti-guinea pig γ globulin (R/GPγ). The R/GPγ was prepared in rabbits, which were sensitised to purified ((NH₄)₂SO₄ precipitation followed by three passages through DEAE cellulose columns) guinea pig γ globulin (GPγ) in Freund's complete adjuvant. It was purified by binding to and elution from GPγ coated cyanogen bromide-activated sepharose beads (Pharmacia). The method of Campbell and Grey was used to coat the plastic beads⁷. A flow rate of 2-2.5 ml min⁻¹ was used and approximately 2 × 10⁹ spleen cells (viability 50-60%) were applied to each of the 30 × 1.5 cm columns. There was a 12-20% recovery of cells from each column. A direct immunofluorescent (IF) technique using either FITC labelled goat or R/GPγ (Nordic) was used to identify B cells. The effluent cells were pooled and found to be depleted of 90-95% of the 27-36% of IF positive cells which were present in the original cell suspensions applied on to the columns. Effluent cells did not stain with FITC-labelled goat anti-rabbit γ globulin which indicated that the cells were not coated with R/GPγ when they were passed through the columns. Plastic bead columns coated with normal rabbit serum (NRS) were used in control experiments. Since one-third of the 3 × 10⁸ viable donor cells were IF-positive in the original suspensions, we transferred 2 × 10⁸ viable effluent (B-cell depleted) cells into the CY treated recipient animals and assayed their ability to suppress the delayed hypersensitivity reactions.

Table 1 Skin reactivity of animals, treated with CY 3 d before sensitisation with 1 µg OA in FIA, serving as cell recipients 8 d after immunisation

Donor cell†	No. of recipients	Increase in skin thickness*		
		24‡	48	72
Control§	11	4.4 ± 0.74	5.6 ± 1.04	4.0 ± 1.10
Unseparated	5	1.8 ± 0.27††	2.2 ± 0.27††	1.4 ± 0.89††
Separated¶	6	4.6 ± 0.58	5.4 ± 0.86	4.3 ± 1.29
(R/GPγ column)				
Unseparated**	5	2.1 ± 0.65	3.1 ± 0.89	2.0 ± 0.79
(NRS column)				

*10⁻¹ mm ± s.d.

†Pooled spleen cells from animals immunised with 1 µg OA in FIA 8 d earlier.

‡Time after intradermal skin test with 100 µg OA.

§Received no cells.

||3 × 10⁸ viable, containing 27-36% IF-positive cells.

¶2 × 10⁸ viable, containing < 3% IF-positive cells.

**3 × 10⁸ viable, containing 26% IF-positive cells.

††P < 0.001 when compared to control animals and separated cell recipients using Student's *t* test.

Table 1 shows that cells that were not passed through the columns suppressed reactions of the recipient CY-treated animals to a similar extent as previously described¹. When cells depleted of 90-95% of B cells (after passage through R/GPγ coated columns) were transferred into identically treated recipients, however, there was no suppression of the delayed-in-onset skin reactions (Table 1). The cells passed through NRS coated columns contained 26% B cells (compared to 28% in the original cell suspension) and suppressed

delayed reactions of the CY-treated recipients to a similar degree as the unseparated cells (Table 1).

We had previously demonstrated that the passive transfer of spleen or PEC from animals sensitised to 1 µg OA in FIA 8 d earlier caused more intense and more indurated reactions in normal recipients when donors had been pre-treated with CY 3 d before sensitisation, as compared to those reactions transferred from sensitised animals which had not received CY (ref. 1). We therefore passively transferred spleen cells, which had been depleted of B cells (as above), into normal guinea pigs to determine whether they would also transfer the more intense and more indurated reactions. Table 2 shows that 2×10^8 viable column-separated, B cell-depleted, spleen cells from animals sensitised with OA in FIA 8 d earlier caused more indurated reactions than the 3×10^8 viable unseparated spleen cells from the same cell pool. We favour the interpretation that B cells are suppressing the expression of delayed hypersensitivity reactions because after removing B cells, the same number of transferred non-B cells were causing more indurated reactions in normal recipients but no longer suppressed the skin reactions in the CY-treated sensitised recipients.

Table 2 Skin reactivity of normal recipients receiving pooled spleen cells from animals immunised with 1 µg OA in FIA 8 d earlier

Donor cells	No. of recipients	Increase in skin thickness*		
		24†	48	72
Unseparated‡	6	2.4 ± 0.58	0.42 ± 0.58	0
Separated§	5	3.9 ± 0.65	2.3 ± 0.57	1.0 ± 0.61

* 10^{-1} mm $\pm$ s.d.

†Time after intradermal skin test with 100 µg OA.

‡ 3×10^8 viable, containing 27–36% IF-positive cells.

§ 2×10^8 viable, containing < 3% IF-positive cells.

|| $P < 0.001$ when compared to control animals and separated cell recipients using Student's *t* test.

These experiments demonstrate that, after immunisation with OA in FIA, B cells or their product(s) suppress the expression of the delayed hypersensitivity reactions. These reactions are undoubtedly T-cell functions because, in the absence of B cells, they are more indurated and more intense. Other recent studies have also demonstrated B-cell suppression of T-cell functions^{8–10}. It should be mentioned that numerous published studies have demonstrated that T cells may amplify, as well as suppress, other T cell functions^{11–14}. It seems therefore that B cells, as well as T cells, are important in the homeostasis of T-cell functions.

The mechanism of the suppression of T-cell functions by B suppressor cells remains unknown. We, as well as others cited above, have been unable to implicate the antibody product of B cells in the mechanism of the suppression. It could be that the suppressor cells in delayed hypersensitivity form a separate subpopulation of easily identifiable Ig-coated cells that is distinct from those which become antibody producers. Effective methods for recovering pure B cell populations should help elucidate the mechanism(s) involved in this suppression.

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New method for extending the diffraction pattern from protein crystals and preventing their radiation damage

A CONSIDERABLE problem hampering the analysis of protein crystal structures has been the damage caused to the crystals by the X-ray beam. Another limiting factor has been the resolution in the diffraction pattern. To increase the useful life of a protein crystal in the X-ray beam¹, single crystal monochromators and low temperatures have been used. We now report that styrene and other vinyl monomers can decrease the radiation damage of the crystal in the X-ray beam by a factor of as much as 10, and extend the diffraction pattern so that higher angle reflections can be measured.

We used crystals of an intact IgG immunoglobulin molecule (DOB) whose crystal structure has been determined to a resolution of 6 Å (ref. 2). These crystals are extremely sensitive to radiation, and intensities of even very low order reflections with d spacings greater than 8 Å decrease to 80% of the starting value in about 15 h of exposure. The diffraction pattern does not show any meas-

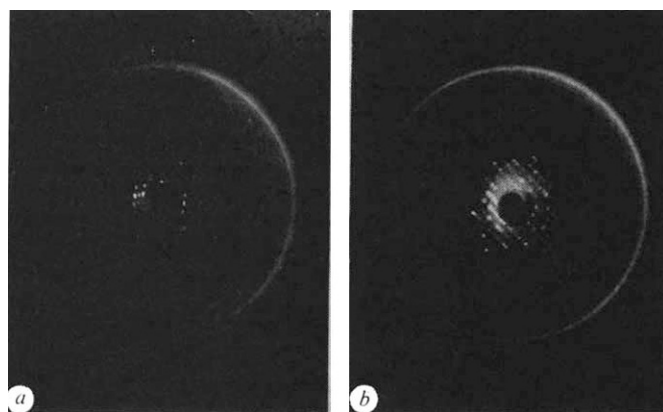


Fig. 1 Diffraction pattern from (a) an untreated and (b) styrene-treated immunoglobulin crystal. The precession angle is 15°, exposure time 45 h. Both crystals were of comparable size. The X-ray beam was parallel to the *b* axis.

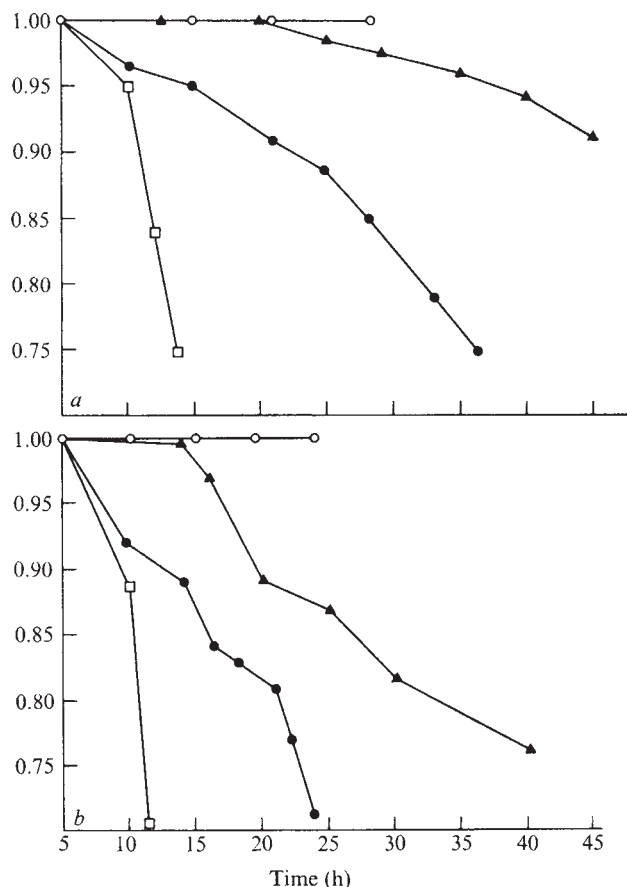


Fig. 2 Variation of I/I_0 with exposure time for two reflections (0 8 0) (a) and (-12 0 6) (b) with the following styrene concentrations: ●, control; ○, 2.0 mM; ▲, 1.2 mM; □, 30 mM. These are styrene concentrations in the protein solution at the start of crystallisation. The actual concentration of styrene inside the crystal lattice is unknown. I_0 corresponds to the background corrected intensity at time = 5 h. A single crystal monochromator was used on the diffractometer and all measurements were carried out at room temperature.

Table 1 Intensity values for a typical row of reflections with decreasing of spacing

h k l	d(Å)	2 mM styrene treated (I_s)	No styrene control 1 (I_1)	No styrene control 2 (I_2)	I_s/I_1	I_s/I_2
9 1 0	20.3	9,287	2,931	6,299	3.1	1.5
11 1 0	16.8	11,054	3,335	7,183	3.3	1.5
13 1 0	14.3	3,819	1,355	2,226	2.8	1.7
15 1 0	12.4	6,497	1,935	3,406	3.3	1.9
17 1 0	10.9	190	0	0	∞	∞
19 1 0	9.8	291	18	141	16.0	2.0
23 1 0	8.1	280	82	142	3.4	2.0
25 1 0	7.5	375	0	223	∞	1.7
29 1 0	6.5	1,390	410	703	3.4	2.0
31 1 0	6.0	613	177	288	3.5	2.1
33 1 0	5.7	199	45	70	4.4	2.8
35 1 0	5.4	233	53	78	4.4	3.0
37 1 0	5.1	504	0	156	∞	3.2

The treated crystal had dimensions $0.8 \times 0.5 \times 0.15$ mm. The control crystal 1 had dimensions $0.7 \times 0.5 \times 0.15$ mm, comparable with the size of the treated crystal. The control crystal 2 was larger than the treated crystal and has dimensions $1.0 \times 0.6 \times 0.15$ mm. The increase in the ratio for the high order reflections suggests a lowering of the temperature factor for the treated crystal. The zero intensities represent those reflections for which the peak to background ratio was less than one. A single crystal monochromator was used on the diffractometer and all measurements were carried out at room temperature.

Results similar to those described above were obtained, but the concentration of the styrene in the protein drop was not so well determined as in the cocrystallisation experiments. We have also used monomers such as chlorostyrene, and methyl methacrylate with similar results. We have not used any styrene-treated crystals for preparing isomorphous heavy atom derivatives.

We propose to calculate a difference electron density map between the treated and the untreated crystal to locate regions of styrene binding in order to understand the actual mechanism of the protection provided by styrene. We speculate that styrene monomers are trapped in the intermolecular volume of the crystal lattice and are polymerised by the X radiation. The aromatic nucleus in styrene is a well known energy trap⁴ and presumably scavenges the free radicals formed in the crystal, thereby reducing its radiation damage. We also suggest that at low styrene concentrations, a polystyrene cage surrounds regions of the protein molecule limiting its movement, thus extending the diffraction pattern. But with very large concentrations of styrene, the rapid polymerisation combined with the increased concentration of polymer presumably gives rise to large disordered regions in the protein molecule thus worsening the observed diffraction pattern.

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urable reflections beyond a resolution of about 5.5 Å (ref. 3) (Fig. 1a).

These crystals are usually grown at 4° C in 0.1M sodium borate buffer at pH 8.8. We have grown the same crystals in the presence of various amounts of concentrated styrene monomers added to the protein drop before it is set for crystallisation. When irradiated, the treatment crystals continued to diffract even after 150 h in the X-ray beam showing sharp reflections that extend to a resolution of 4 Å (Fig. 1b). Due to increased error involved in adding less than 0.5 μl of styrene directly to the protein drop, we also crystallised the immunoglobulin by adding different amounts of the buffer saturated with styrene.

Figure 2 shows the variation in intensity with time of exposure of two arbitrarily chosen reflections from an untreated crystal and crystals treated with styrene. Data were collected using a Nonius CAD4 automatic diffractometer. Table 1 shows the intensities of a typical row of reflections with decreasing interplanar spacings from two control crystals of different dimensions and one treated crystal. The intensities from the treated crystal were greater than those from the control crystal even when the latter had a volume 50% greater than the former. This effect was particularly noticeable with the higher order reflections.

We have diffused styrene monomers into pregrown immunoglobulin crystals by placing a drop of the concentrated styrene adjacent to the protein drop in a sealed well.

Do immunologically privileged sites require a functioning spleen?

WHEN solid tissue allografts are transplanted orthotopically to alien hosts they are rejected with a characteristic tempo and vigour that depends primarily on the immunogenetic disparity between donor and host. There are, however, several anatomical sites in which prolonged graft survivals have been documented. These so-called immunologically privileged sites include the brain^{1,2}, anterior chamber (AC) of the eye³⁻⁵, cheek pouch of the Syrian hamster⁶ and artificially created skin pedicle flaps in guinea pigs and rats^{7,8}. There is considerable experimental evidence that these sites share at least one common feature—absence of a demonstrable lymphatic drainage⁷⁻⁹. As a consequence, it has been assumed that the putative immunological privilege of these diverse sites results from the anatomical aberration which interrupts the afferent limb of the immunological reflex arc (which is composed of an afferent limb, a central mechanism and an efferent limb). We have now found that allogeneic cells placed within the AC of the eye are not isolated from the immunological apparatus of the host, but instead induce a primary immune response within the spleen which alters the systemic immunological response of the intact animal.

To determine the status of a lymphatic drainage route from the AC, we used a local graft-versus-host lymph node assay. Lymph node cells from Fischer rats specifically sensitised to tissue antigens of DA strain rats were inoculated into either the AC or the subconjunctival space (SCS) of (F×DA)F₁ recipients. Eight days later (Table 1) hypertrophied cervical lymph nodes developed in response to SCS injections, whereas local lymphadenopathy did not follow AC injections of donor cells. This provided strong circumstantial evidence that the AC lacks lymphatic drainage.

Table 1 Comparison of cervical lymph node hypertrophy after AC or SCS inoculation of allogeneic lymphoid cells

Route of inoculation	No. of animals	Experimental*(mg)†	Control	P
Subconjunctival space	10	237.2	84.2	<0.005
Anterior chamber	22	88.0	89.4	>0.20

*Lymph nodes to be weighed were removed from (F × DA)F₁ hybrid hosts 8 d after the inoculation of 10×10^6 F anti-DA lymph node cells into the anterior chamber or subconjunctival space.

†The weights of the superficial cervical lymph nodes, that is the submandibular and facial nodes, are reported.

Slit lamp microscopy showed that after being placed in the AC the inoculated cells disappeared within a few days by an unknown route. These cells reached the systemic circulation and made a significant impact on the host's immunological apparatus, as our next series of experiments showed. In the first, genetically tolerant (F×DA)F₁ lymphoid cells were inoculated into the AC of normal Fischer rats whose sera were regularly tested for haemagglutinating activity directed at DA alloantigens. Surprisingly, anti-DA antibodies were identified as soon as 4 d after inoculation and persisted for at least 10 d thereafter (Fig. 1). In the second series of experiments, panels of Fischer animals were inoculated similarly with (F×DA)F₁ lymphocytes into the AC and challenged orthotopically with (F×DA)F₁ skin allografts 4, 7, 10 and 14 d later. During the second week after inoculation the animals exhibited significantly prolonged survival of (F×DA)F₁ hybrid skin grafts (Fig. 2). The median survival time (MST) of F₁ skin grafted 10 d

after inoculation was 12.0 d, with some grafts surviving 14 d. Since first set (F×DA)F₁ skin grafts were rejected by normal hosts with a MST of 9.1 d, the prolonged MST in the experimental group during the second week is highly significant. It is interesting that F₁ skin grafts applied during the first week after inoculation had a MST similar to that of first set grafts. The similarity of MSTs for first and second set skin allograft rejection in rats with an AgB histoincompatibility makes it very difficult to distinguish between the two. Consequently, it is possible that during

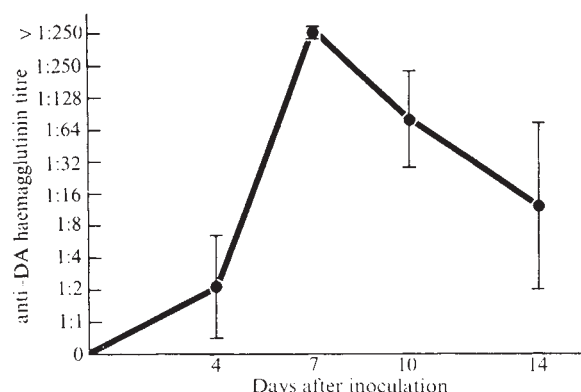


Fig. 1 Anti-DA haemagglutinin antibody titres after the inoculation of 10×10^6 (F×DA)F₁ lymphoid cells into the AC of Fischer hosts ($n=6$). Bars represent one standard deviation.

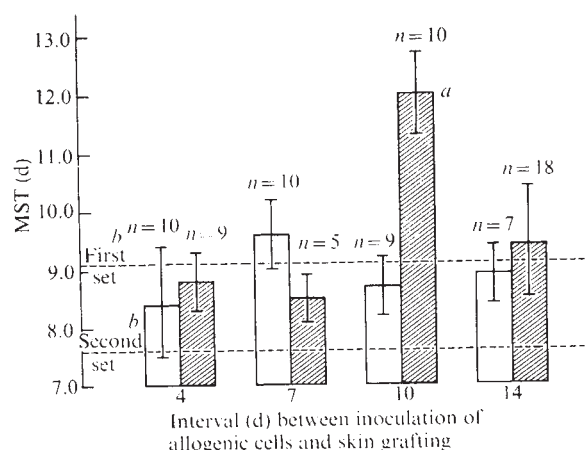


Fig. 2 The comparative MSTs of (F×DA)F₁ hybrid skin grafted within the first 2 weeks after inoculation of 10×10^6 (F×DA)F₁ LNC into the AC (hatched columns) or subconjunctival space (open columns) of Fischer hosts. The 95% confidence limits are represented. *a*, The MST of F₁ skin allografts 10 d after inoculation of allogeneic lymphoid cells into the AC is statistically significant from a first set reaction, using Student's *t* test, with $P < 0.001$. *b*, MSTs of first and second set (F×DA)F₁ grafts in normal Fischer hosts are presented for comparison.

the first week after inoculation of F₁ lymphoid cells into the AC, some recipients were sensitised specifically without its being revealed by the gross test of graft rejection.

By contrast, grafts of F₁ origin placed on Fischer hosts that had been inoculated subconjunctivally with (F×DA)F₁ lymphoid cells were rejected abruptly irrespective of the time between inoculation and graft application (Fig. 2).

Only intravenous antigen presentation gave results similar to those obtained with injection into the AC (Fig. 3).

Since the AC lacks lymphatic drainage, and since our data suggest that alloantigens on lymphocytes placed in the AC made an impact on the host's immunological apparatus, we suggest that donor lymphoid cells escaped from the AC directly into the blood vasculature of the host. By virtue of its size and blood flow, the spleen receives the majority of antigen presented to an animal intravenously¹⁰⁻¹². Moreover, the spleen has been identified as a major source of enhancing antibodies¹³. Consequently, it seemed reasonable to determine whether the facilitation of skin graft survival displayed by Fischer hosts during the second week after inoculation of (F×DA)F₁ cells, by the AC or intravenous routes, depended on an intact, functioning spleen. Accordingly, Fischer hosts were splenectomised and after 28 d were inoculated, into the AC or intravenously, with (F×DA)F₁ lymphocytes. Challenge with skin grafts of (F×DA)F₁ origin (Fig. 3), revealed no evidence of enhancement or facilitation; in fact, the MST of these grafts (8.3 and 7.8 d respectively) approached the accelerated rejection usually found in specifically sensitised hosts (7.8 d). These data suggest that the spleen is an essential component of the immunological mechanism that temporarily subverts cell-mediated responses.

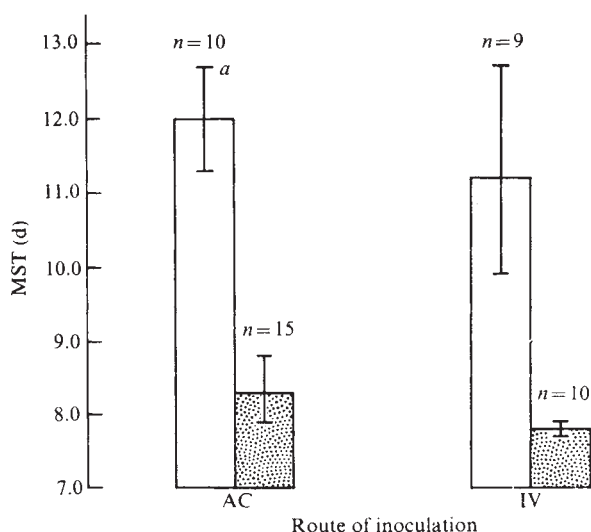


Fig. 3 A comparison of the MSTs of (F×DA)F₁ hybrid skin grafts in normal (open columns) and splenectomised (dotted columns) Fischer hosts after the inoculation of 10×10^6 (F×DA)F₁ lymphoid cells into the AC, 10 d before grafting, or intravenously, 7 d before grafting. The shortened survival time of allografts on splenectomised hosts, after both AC or intravenous injection of antigen, is highly significant compared with normal hosts ($P < 0.0005$). a, The 95% confidence limits are represented.

Skin grafts placed within the AC exhibit survival patterns significantly different from orthotopically placed grafts, which cannot be attributed solely to a delay in host sensitisation^{4,20}. We propose that although all immunologically privileged sites appear to lack demonstrable lymphatic drainage routes^{7-9,14}, the critical feature for at least some of them is the ability to allow antigen direct access to the blood vasculature without first encountering a peripheral lymph node. As a consequence, the host's initial antigen exposure occurs predominantly within the spleen and our results indicate that the microenvironment of this initial encounter significantly influences the nature of the subsequent immunological response. Our data do

not reveal which components of the spleen give it the special properties we have observed. But, together with other results¹⁵⁻¹⁹, they suggest that one immunological function of the spleen is to divert what would otherwise be essentially a cell-mediated and tissue destructive response into an antibody-mediated response which is often protective of the same target tissue. It seems reasonable to speculate that the spleen achieves this conversion by deflecting potential effector T lymphocytes (killers) into cooperative interactions (helpers) with appropriate B cells. An effective cell-mediated response is thus thwarted by (1) a temporary reduction in the number of circulating killer cells and (2) an enforced competition between the remaining killer cells and enhancing antibodies directed at the same target tissue.

It is conceivable that this immunoregulatory role of the spleen in the induction of the immune response represents the evolutionary advantage of vertebrates in coping swiftly and successfully with certain kinds of hazardous microbial infections²¹⁻²⁴. By the same token, however, it may place the host at considerable disadvantage with regard to the capacity to control aberrant and neoplastic growth for which cell-mediated immune processes are crucial.

We thank R. E. Billingham for helpful discussions and critical review, R. Zeppa for support, and R. G. Holt for technical assistance. This work was supported by a National Institutes of Health research grant and research training fellowship.

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reviews

"I BEG to apply for the Poynting Professorship of Physics . . . I have been nominated for and hope to obtain a temporary commission in the regular army preferably in the Royal Engineers. My services are not therefore likely to be available while the war lasts." So wrote H. G. J. Moseley, then 26 years old, to the University of Birmingham on October 8, 1914. On August 10, 1915, he was killed in a desperate action against the Turks in Gallipoli.

With a father and grandfather both Fellows of the Royal Society, with an Eton and Oxford education and a private income, Moseley was, as his American biographer shows, a very English physicist. In his short life he had already accomplished one of the most brilliant pieces of experimental physics of this century, and he was a hero to the schoolboys of my generation who learnt, as E. J. Holmyard put it in his textbook of Chemistry (quoting Soddy), that Moseley had "called the roll" of the chemical elements and put them all in their place.

Moseley's example was a comfort, too, to the undergraduates of my day, for he had taken only Second Class Honours in Physics Finals at Oxford. At an Oxford Physical Society dinner in 1931 I was sitting next to our Professor, J. S. E. Townsend, and asked him whether this was so; Townsend told me how indignant he had been that Moseley had been given a Second, and said that if he had known that Moseley was ill (from hay fever) he would have remonstrated with the examiners before the results were published.

That was in 1910; after finals Moseley went to Manchester, where he had been attracted by the brilliance of Rutherford. The latter put him on to determining the number of electrons emitted in the decay of radium, a task which he completed in April 1912. In that same month, Friedrich and Knipping, prompted by von Laue, discovered the diffraction of X rays, and the work was taken up in Leeds by the Braggs. Barkla had already discovered that elements under electron bombardment emitted two characteristic rays, one harder than the other, which he designated K and L, and Whiddington had found in 1911 that the velocity which cathode rays must have to excite K radiation in an element of atomic weight A is about

Some corner of a foreign field

by R. V. Jones

H. G. J. Moseley: The Life and Letters of an English Physicist, 1887-1915. By J. L. Heilbron. Pp. viii+312. (University of California: Berkeley, Los Angeles and London, June 1974.) £7.50.

$A \times 10^8 \text{ cm s}^{-1}$. With the debate going on between the Braggs, von Laue and Barkla concerning the nature of X rays, Moseley became interested; and together with C. G. Darwin he started to experiment with them. (But could he have used a mercury vapour pump in 1912? (page 73)—Gaede did not describe such a pump till 1915.)

Moseley's skill with apparatus quickly resulted in his recognition of X-ray wavelengths as characteristics of atomic structure and, as he wrote to his mother in May 1913, "there is here a whole new branch of spectroscopy, which is sure to tell one much about the nature of an atom." He was soon recording the X-ray spectra of various elements, and by the following November he had found that the frequency of the K_α line is given by

$$\nu_{K_\alpha}/\nu_0 = (3/4)(Z-1)^2$$

where ν_0 is the Rydberg frequency already known from optical spectroscopy, and Z the atomic number; and he equated the atomic number with nuclear charge which he recognised as chemically more significant than atomic weight. At that point he returned to the Electrical Laboratory in Oxford, where he completed the work on some 30 elements and was able to put all these elements in order, including some hotly disputed rare earths, simply by determining the atomic numbers from the K_α frequencies. Moreover, he could show exactly where any gaps remained to be filled in the Periodic Table, and he could make sense of those few elements which, judged by their atomic weights, had seemed to be out of place.

By 1914 Moseley was interested in the prospectively vacant chairs in Oxford and Birmingham; but war broke out just as he was about to land in Australia for the British Association meeting. He made a

rapid return to England with Henry Tizard after the meeting, both to volunteer for active service. They left ship at San Francisco to cross America by rail, and ran to catch the Lusitania in New York, arriving in England on September 28. After a period of military training with the Royal Engineers (he had been in the OTC at Oxford) Moseley volunteered for the Royal Flying Corps; but his transfer was vetoed, and he was sent to Gallipoli.

On the way out he made a will bequeathing all his interests to the Royal Society "to be applied to the furtherance of experimental research in Pathology, Physics, Physiology, Chemistry, or other branches of science but not in pure mathematics, astronomy, or any branch of science which aims merely at describing cataloguing or systematizing". This bequest formed the basis of the Moseley Studentship, whose second holder was P. M. S. Blackett.

Moseley had been one of the 15 or so King's Scholars elected to Eton in 1901: six of them "including all who were natural leaders in work or play" were killed in the war. Moseley's death, recognised even in Germany for the loss it was to science, was more than any other event instrumental in our wiser use of scientific talent in the Second World War.

The story of Moseley's life is admirably told by Professor Heilbron, who has been to great pains to gather Moseley's letters and to investigate many private papers of his contemporaries. The letters are both piquant and poignant: they give impish sidelights on figures such as Hugh Dalton, Harold Hartley, Julian Huxley, Lindemann, Rutherford, Tizard and Townsend, and show both the attractions and defects of Edwardian Oxford with its overpowering classical complacency. As Professor Heilbron speculates in his epilogue, the development of physics at Oxford would probably have been different if Moseley had survived, for he would probably have been preferred over Lindemann, and he would have been more active in the laboratory and a Nobel prize winner.

Though physics is no medium for nationalism, the sub-title of this volume is significant for more than the fact that in connection with Moseley it must recall the lines of *The Soldier* by his contemporary Rupert Brooke, who

was also to die in the Gallipoli campaign. For Moseley himself wrote to Rutherford "the French point of view [of physics] is essentially different from the English. Where we try to find models or analogies, they are content with laws." But irrespective of whatever truth there may have been in this comment, physicists of all nationalities who are interested in the development of their subject owe Professor Heilbron a debt for his excellent biography. And if this review seems to have been coloured by Moseley's own opinion that "reviews in *Nature* are usually such pure honey", in this case both the subject and his biographer deserve it.

Evolution of populations

Genetical Structure of Populations. By Kenneth Mather. Pp. viii + 197. (Chapman and Hall: London, 1973.) £4.00.

PROFESSOR MATHER'S latest book is a survey of topics in population genetics, intended for advanced undergraduate and postgraduate students seeking a general orientation. The subject is the way in which proportions of genetic types change from generation to generation in response to various factors, and the effect of these changes on the status of the population in relation to its environment. The approach is quantitative but not mathematical, being directed to readers with biological education and interests. The mathematical level varies considerably from chapter to chapter with more extensive algebraic treatment of traditional topics such as the Hardy-Weinberg law and its generalisations.

After a short introduction the style of the work is established in the second chapter which reviews the causes of variability in biology. Professor Mather assumes that readers understand the basic facts of inheritance, and devotes himself to a detailed exposition of the way in which these affect population changes. Although the results of algebraic analysis are stated, most proofs are omitted. Later in the book, chapter 6 on "Life Cycles and Genetic Systems" is a mine of interesting information but contains no mathematical analysis at all. Chapter 7 on "Genic Balance and Genetic Architecture" involves quantitative statements about the population effects of the manner in which the genetic system acts on numerical characteristics, but again contains no proofs.

Chapters 3 and 4 ("Equilibria" and "Theory of Variability") reveal the strength and weakness of the attempt to discuss a quantitative subject while eschewing advanced mathematics. In mathematical books on population genetics (among which Moran's *Statistical Processes of Evolutionary Theory*,

Reproduction of marine invertebrates

by R. P. Dales

Reproduction of Marine Invertebrates. Vol. 1: Acoelomate and Pseudocoelomate Metazons. Edited by Arthur C. Giese and John S. Pearse. Pp. xi + 546. (Academic: New York and London, 1974.) \$38; £13.45.

THIS useful and scholarly work is the first of seven volumes. The first six will give a systematic treatment with a chapter devoted to each class or phylum; the final volume will be devoted to general aspects of reproductive physiology. This first volume is especially useful for its coverage of so many minor but interesting phyla such as Kinorhynchs and Gnathostomulids. But all of the chapters are useful: I found the amount of descriptive detail to have been well judged and the accounts of reproduction and embryonic development to be concise and informative. The appearance of the series is timely for there has been much recent work on reproduction in invertebrate phyla.

References are given in full at the end of each chapter and the volume as a whole is provided with separate indices to authors, subjects and taxonomic names. The volume, containing many line figures and half-tones, is well produced. No zoological library can afford to be without this series.

published in 1962 remains unsurpassed despite many subsequent competitors) the problem of selection, random tendency to fixation, mutation, non-random mating and so on, are treated as separate topics. In nature they are all acting simultaneously, one reinforcing or counteracting the effect of another. Professor Mather makes this clear by detailed examples in a full and well balanced discussion. This chapter leaves the reader with a profound respect for the author's deep knowledge of what is really going on in a natural population and a realisation of how much can be lost through oversimplification.

Two points of substance are worthy of note. It is clear towards the end that Mather believes that competition is always frequency dependent, and it must follow that much recent work in mathematical genetics is of little value since it assumes fixed selection coefficients. He devotes several pages to a discussion of the concept of 'genetic load' which has been so hotly debated in recent years. Mathematicians with an interest in

genetics, who have been worried by their inability to see the point of all this, will be pleased that such a distinguished geneticist concludes "it is far from clear that the concept, as it has been defined and used, has much significance at all for the understanding of populations and their structure".

In discussing the theory of variability, Professor Mather applies a statistical analysis of variance to the variation in a numerical trait in a population. He supposes, however, that it contains equal proportions of the alleles at one locus or at each of two loci, and obtains components of free, potential and utilised variability which can be transformed into one another as a result of various effects and which are "acted on" by the factors of selection. These ideas clearly give insight into the heart of Mendelism, the fact that variability provides the basis for evolution. They would explain it completely if, when the gene proportions are not equal, the transformation of components behaves qualitatively in the same way. Now in the case of constant selection depending on two or more loci this is not true. There may be several stable equilibria, each with its domain of attraction, and it is unlikely that the same analysis of the responses of components of variability to selective factors will hold in the neighbourhood of them all. The analysis is therefore too restricted in its validity to be convincing. The weakness of this chapter and the previous one is the readiness with which it is assumed that the qualitative results of algebra applied to simple systems can be extended to complex systems for which results have not been obtained.

This has led to wrong conclusions in the past, and the student should be warned against the practice rather than led into it. It may well be that when it becomes possible to analyse fully realistic models, the results will prove to be on the lines described by Professor Mather on the basis of his wide knowledge, since factors neglected in (say) the multilocus constant selection model may then appear and eliminate superfluous stable equilibria. It is also probable that a satisfactory mathematical analysis of these problems cannot be achieved through classical algebra and will require the application to genetics of topological considerations such as those introduced by Thom in biology.

This is an admirable textbook for students, containing a masterly exposition of what is really important in our current knowledge of population genetics. In order to go beyond it a student will need to study the mathematical side of the subject at a much higher level, and should be encouraged to regard at least some of the assertions as tentative until they have been proved.

PHILIP HOLGATE

THE authors define sub-clinical lead poisoning as the production of morbidity or mortality without the appearance of the classical signs or symptoms of lead poisoning. This is a highly emotive subject and the authors are to be congratulated for dealing with it in a balanced and scientific manner. They have provided an excellent review of the literature including that from eastern Europe, and over 1,000 references are given together with an author's index.

The book is divided into six main sections: lead in the environment, lead metabolism, pathological effects

Dangers of lead

by B. E. Clayton

Sub-Clinical Lead Poisoning. By H. A. Waldron and D. Stoffen. Pp. x+224. (Academic: London and New York, May 1974.) £5.50; \$14.25.

of lead, sub-clinical effects, diagnosis and lastly prophylaxis and treatment. With such a controversial subject it is not surprising that I do not agree with all the statements made but the authors' main conclusion would be accepted by most workers. They consider that more studies are required

before it is possible to assess whether or not excessive exposure below that causing clinical lead poisoning is producing harmless physiological or psychological effects, especially in children.

The book is well produced. The list of references would have been even more valuable if titles of papers had been given. An appendix listing quantities of lead in food and drink is included. It is written clearly and workers from various disciplines would find it valuable. I recommend it to all those concerned with this difficult environmental problem.

Natural death

Biological Control. By C. B. Huffaker. Pp. xix+511. (Plenum: New York and London, 1974.) \$11.75.

IN recent years, increasing concern at the possible pollution of the environment by chemical pesticides has directed attention back to studies of non-chemical methods of pest control. Before DDT and other efficient synthetic insecticides were brought into general use in the 1940s, control of pests by biological means, particularly by parasitic or predatory insects, had resulted in many successes. Some of these, like the classical case of the Australian ladybird *Rodalia cardinalis*, which, in 1877, controlled the cottony cushion scale *Icerya purchasi*, have never been bettered, but there have been many other successes, some partial control, and some complete failures. Nevertheless, as F. J. Simmonds has shown, the investment in research was so paltry, and the results in increased crops were so great, that the whole exercise, on a world scale, was extremely cost-effective. The trouble was often that the individual who gained could not easily be implicated in the costs of the control. Commercially produced chemical pesticides, on the other hand, could bring their financial rewards to industrial concerns.

This paperback reprint of the proceedings of a conference, aims at making up to date information about biological and other methods of pest control available to a wider audience, and particularly to university students of entomology, agriculture and allied subjects. If read critically, it should form a useful text.

Nobody doubts that, particularly when improperly used, some agricultural chemicals can endanger wildlife, the environment and even man himself. We would all like to reduce the use of dangerous chemicals, and to use non-polluting methods of pest control. But, unfortunately, some of the authors whose articles are reprinted here tend to overstate their

case. Thus the editor, C. B. Huffaker, says in his preface "employment of synthesised chemicals for insect pest control has posed an enormous and as yet unfathomed contribution to the degradation of our environment". This, and other similar statements, conceal the fact that over ninety per cent of pesticide usage throughout the world is effective, in that it achieves its object and no measurable damage can be demonstrated. Yet we are clearly right to worry about potential dangers, and to try to find more effective controls.

This book differs from previous texts on biological control in that although it describes once more all the well known successes, it includes a section on the ecological background to the subject and also devotes considerable space to 'integrated control' or what is now often called 'pest management'. G. C. Varley and G. R. Gradwell deal with the fundamental basis in population ecology in their paper "The Use of Models and Life Tables in Assessing the Role of Natural Enemies". An understanding of these matters is obviously essential if biological control is to cease to be a hit and miss affair. Unfortunately, these authors are still unable to demonstrate a single case where theoretical or mathematical studies have given any practical help to those engaged in practical control measures. The inspired hunch has, on the other hand, often paid off. It is to be hoped that this will not discourage others from these fundamental studies, which are necessary long-term—and which, it must be accepted, may never produce the desired results.

The main lesson the book teaches is that put forward many years ago by the late W. E. Ripper: that is that an "insecticide should be used like a rapier, not like a bludgeon". This is the essence of integrated control—natural enemies of pests should be encouraged but when they fail the correct chemical may be carefully used. Also we are now learning that total pest eradication is neither essential nor de-

sirable. Pests can often be 'managed' to prevent patent crop damage, and, at the same time, so as to ensure the survival of their natural enemies. All these techniques will undoubtedly increase in importance, particularly if premature bans on allegedly dangerous chemicals continue to be introduced in some countries. But the farmer, and even more the medical entomologist, will rely mainly on chemicals for many years to come. It is right, however, that the disadvantages as well as the gains from chemical control should be recorded, though few writers can yet take a really objective view of the situation.

KENNETH MELLANBY

1975 Catalogue of SCIENTIFIC & TECHNICAL JOURNALS FROM THE U.S.S.R.

on

GEOLOGY, GEOPHYSICS, BIOCHEMISTRY, BIOPHYSICS, NATURAL SCIENCES

and related subjects
(in Russian but many with
summaries in English)
may be obtained from

Collet's

**Denington Estate
Wellingborough, UK**

obituary

B. L. Astaurov

ACADEMICIAN BORIS L'VOVICH ASTAUROV, the eminent Soviet geneticist, died on June 21, 1974.

Astaurov was born in 1904, and was educated at the University of Moscow, where he studied under S. S. Chetverikov, the founder of the Soviet school of experimental genetics. Shortly after graduation, he began working on the heredity and genetics of silkworms, completing his first major work in 1937. A convinced anti-Lysenkoist, whose career suffered considerably during the years of Lysenkoism, Astaurov became a focus for the anti-Lysenkoist reaction. His 60th birthday party became a celebration of the fall of Lysenkoism and Astaurov played a significant part in the rebirth of Soviet genetics. He was elected President of the 'Vavilov' All-Union Society of Geneticists and Breeders in 1966.

M. Reinhard

PROFESSOR MAX REINHARD, a leading authority on Feldspars, died on August 17, 1974. He was 92.

After acting as an assistant to Professor Duparc at the University of Geneva, he was appointed to the Chair of Mineralogy and Petrology at the University of Basel in 1923. He became Rektor of the University in 1943 and retired in 1952 with the title Professor Emeritus.

Announcements

Miscellaneous

United States Public Health Service International Postdoctoral Research Fellowships. Up to six awards may be made to Australians for research training in 'health-related' fields in the USA. Applications to the Executive Secretary, Australian Academy of Sciences, PO Box 216, Civic Square, ACT 2608, Australia.

The Worshipful Company of Scientific Instrument Makers/MIT Traveling Fellowship. The Fellowship is open to a suitably qualified graduate with appropriate experience who must be a British national. Full details and application forms are obtainable from the Clerk of the Worshipful Company of Scientific Instrument Makers, Alliance House, 12 Caxton Street, London W1H 0QY.

International meetings

October 14-18, **Air Quality Management Systems**, Milan (Richard Tudway, International Institute for the Management of Technology, 59-63 Corso Magenta, Milan, Italy).

October 24, **Natural Products in Aquatic Environments**, London (Dr John F. Gibson, The Chemical Society, Burlington House, London W1V 0BN).

Corrigendum

In the article "Exchange of neurotransmitter amino acid at nerve endings can stimulate high affinity uptake" by G. Levi and M. Raiteri (*Nature*, **250**, 735; 1974) the sentence at lines 21-22, p. 737 should read: Net uptake, which may or may not have a high affinity component . . .

Errata

In the article "Interaction of coherent electromagnetic waves with relativistic electrons in a medium" by S. Schneider and R. Spitzer (*Nature*, **250**, 643; 1974) the following corrections should be made. In equation (2) the factor $(\tau)\theta(\gamma\tau-\theta)$ should be replaced by $\theta(\tau)\theta(\gamma\tau-\rho)$. The relationship $\varepsilon=n^2$, three lines above equation (2), should read $\varepsilon\neq n^2$.

In the article "Nitrosoguanidine mutagenesis during nuclear and mitochondrial gene replication" by I. W. Dawes and B. L. A. Carter (*Nature*, **250**, 709; 1974) Figs 1 and 4 were transposed. The legends were given correctly.

Reports and publications

Great Britain

It's Like This . . . 500 Years of Pictures for Science. (City of Nottingham Leisure Services—Nottingham Festival 1974.) Pp. 87. (Nottingham: The Castle Museum and Art Gallery, 1974.) 50p. [257]
University of Oxford. Annual Report of the Curators of the Bodleian Library for 1972-1973. Pp. 51. (Supplement No. 5 to the *University Gazette*, June 1974.) (Oxford: The University, 1974.) £1. [297]
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Medical Research Council: Laboratory Animals Centre. Manual Series, No. 3: Survey of the Numbers and Types of Laboratory Animals Used in the United Kingdom in 1972. Pp. 16. (Carshalton, Surrey: MRC, Laboratory Animals Centre, 1974.) [297]
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